Metabolomics for Natural Products: Fast screening and Discovery

Dissertation

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Chapter 1

General Introduction

General Introduction

1.1 Natural products : Small molecules with big roles

Natural products, the remarkable collection of low molecular weight compounds made by living organisms have been exploited for human use for thousands of years and have made important contributions to organic chemistry, biology, medicine, agriculture and numerous other fields. Throughout history, humans have displayed a great enthusiasm and interest in naturally occurring compounds from microbial, plants and animals sources. Early man realized that many plants contain components with powerful biological effects and thus utilized them as a major resource for treating and preventing diseases. This formed the basis of sophisticated traditional medicinal practices that are still popular among many communities around the world (Clardy and Fischbach, 2008).

After centuries of empirical use of herbal preparations, the first isolation of the isoquinoline alkaloid, morphine in the early 19^{th} century marked a new era in the use of medicinal plants and the beginning of modern medicinal plant research (Samuelsson, 2004). Subsequently, many valuable drugs like atropine, cocaine, codeine, digitoxin, quinine, etc. came into use through the study of indigenous remedies. These discoveries made significant contribution to the development of organic and medicinal chemistry (Sneader, 2005).

The discovery and the development of penicillin as a microbial metabolite was another breakthrough in this field and opened up the era of antibiotics, saving countless lives in the last century. Isolation, concentration, purification and mass production of penicillin was followed by the development of streptomycin, tetracycline, chloramphenicol and many other antibacterial agents whose origin in most cases could be traced to naturally occurring sources (Dax, 1997). Furthermore, the isolation and synthesis of steroid hormones as well as the discovery of bioactive

lipids such as prostaglandins and leukatrienes have made a big impact on modern medicine and also on the development of novel concepts in molecular biology (Ogura, 1997).

Despite the recent interest in other drug discovery approaches such as molecular modeling, combinatorial chemistry, and other synthetic chemistry methods, natural products are still providing their fair share of new clinical candidates and drugs (Butler, 2004). About half of the drugs currently in clinical use are based on natural product scaffolds (Newman and Cragg, 2007; Harvey, 2008). These compounds are derived directly, by use of semi-synthetic natural product analogs, or indirectly-through the use of synthetic compounds based upon natural product pharmacophores. Therefore natural-product-derived compounds are still proving to be an invaluable source of medicines for humans.

Natural products often have an ecological role in regulating the interactions between plants, micro-organisms, insects and animals and act as defensive substances, anti-feedants, attractants, pheromones etc.

The concept of "natural pesticides" arose early in the development of agriculture (Dayan, et al., 2009) and the use of natural product and natural product–derived insecticides continue to increase nowadays. These compounds be touted as attractive alternatives to synthetic chemical insecticides for pest management due to their minimum threat to the environment and to the human health (Isman, 2006). The discovery of botanical insecticidal powders from *Chrysanthemum cinearifolium* flower heads and *Derris* root which contain pyrethrum and rotenone, respectively formed the basis of commercial insecticides (Dayan, et al., 2009). Azadirachtin isolated from the Indian neem tree, *Azadirachta indica* is another well known potent antifeedant to many insects, which could block the synthesis and release of molting hormones leading to incomplete ecdysis in immature insects. (Isman, 2006). Today azadirachtin based products are well established commercially and extensively used as organic insecticides against wide range of insects.

The idea of using naturally-occurring compounds as herbicides has ancient origins, however, natural herbicides have not gained much popularity mainly because of their little or no selectivity as well as the need of being applied in large quantities. Nevertheless the quest for such compounds has taken on renewed vigor in recent years with the concept of organic farming. Several essential oils such as lemon grass oil, clove oil, citronella oil have been commercialized and act as non-selective, contact herbicides. Although "allelopathy" does not involve the direct application of natural products for weed management, specific allelochemicals for example Sorgolene, Momilactone, Benzoxazinoids, etc. have been identified as being the primary molecules involved in weed control by crops (Dayan, et al., 2009).

Biological control of plant diseases and plant pathogens is of great significance in forestry and agriculture. In crop production, over half of potential crop yield is lost due to plant pathogens, and in storage, up to one third of the harvest product can be lost due to post-harvest diseases, mostly as a result of activities of fungi (Prescott, et al, 1996). The excessive use of synthetic chemicals to combat such situations has been counterproductive, causing damage to the environment and humans while the continuing development of fungicide resistance in plant pathogens necessitates the discovery and development of new fungicides which are eco-friendly (Martinez, 2012).

Many natural compounds and preparations have been described with activity against bacterial or fungal plant pathogens and some are available in the market for the management of plant diseases in organic agriculture. Several plant essential oils for example jojoba oil, rosemary oil, thyme oil have been marketed as fungicides for organic farmers while an extract of the giant knotweed (*Reynourtria sachalinensis*) is used in Europe for the control of a broad spectrum of both fungal and bacterial plant diseases in both organic and non-organic agriculture. In addition, fermentation secondary products from actinomycetes (mostly Streptomyces spp.) have been

commercialized and used extensively as agricultural fungicides in several countries (Dayan, et al., 2009).

Natural products often serve central roles as biological signaling agents, usually referred to as "semiochemicals", and have been widely considered within various integrated pest management (IPM) strategies. These compounds are now recognized as performing a multitude of vital functions including serving as quorum sensing agents among bacteria, as algal and fungal gamete attractants, as sex attractants and alarm pheromones in many insect species, as attractants to plant pollinating organisms, as plant and animal defensive chemicals, etc. (Meinwald, 2011).

Among the diverse forms of semiochemicals, pheromones, the compounds involved in intra-specific communications, have been subjected to extensive studies in the recent past. The first isolation and identification of an insect pheromone, bombykol ((10*E*,12*Z*)-10,12-hexadecadien-1-ol) from the female silk worm moth *Bombyx mori* occurred in 1959. However the existence of insect pheromones has been known for centuries, apparently originating in observations of mass bee stinging in response to a chemical released by the sting of a single bee (Flint and Doane, 1996). Since then, hundreds or perhaps thousands of insect pheromones might have been identified. Use of pheromones for pest control promises to be an important component of the ongoing challenge to develop alternatives that could help to solve problems associated with chemical pesticides (Kirsch, 1988). Therefore, understanding Nature's chemical language has a great potential in agriculture.

Taking all the above mentioned aspects together, Nature has created secondary metabolites for a plethora of roles and humans have always inquisitively attempted to harness these benefits. Thus, applications of natural products chemistry have become all-pervasive in modern society and the continuous quest to identify the myriad secondary metabolites demands the availability of highly selective, sensitive, precise, accurate, reproducible and efficient analytical techniques.

1.2. Old tools in natural products chemistry

The complex nature of natural products usually requires maximum performance from the sample preparation, separation and identification methods and these tedious procedures are the main bottle-neck in natural products chemistry (Casas, 2009).

In order to obtain secondary metabolites from biogenic materials, the first step is to release them from the matrix by means of extraction (Cannell, 1998). The choice of extraction method is of great importance as an incorrect approach would not allow the release of all of the desired components. The conventional extraction methods involve the use of solvents, either organic or aqueous while supercritical fluid extraction (SFE) is a more recent method whose application has steadily increased. However, the current trends in extraction techniques have largely focused on finding solutions that minimize the use of solvents (Casas, 2009).

Isolation of natural products generally combines various separation techniques depending on the solubility, volatility and stability of the compounds to be separated. The choice of different separation steps and an analytical-scale optimization of the separation parameters are of great importance for the maximum outcome (Sticher, 2008).

The conventional natural product isolation strategies consist of extremely time consuming and technically demanding multi-step purifications. These involve techniques like preparative thin layer chromatography, conventional column chromatography, flash chromatography, medium pressure liquid chromatography, vacuum liquid chromatography, high performance liquid chromatography etc. often performed with the purpose of obtaining pure compounds for structure elucidation. Once the purification is completed, several spectroscopic techniques such as nuclear magnetic resonance (NMR), ultraviolet spectroscopy (UV), infrared spectroscopy (IR), mass spectrometry (MS), and X-ray crystallography are utilized in the identification and characterization of natural products. As these conventional

approaches require substantial amount of purified compound to solve the puzzle of the compounds' structural identity, the structural characterization of minor components in a bioactive fraction appears unfeasible.

In bio-activity guided fractionation approaches, the major disadvantage is the inherent risk of re-isolating known or trivial constituents. This necessitates the development of new methodologies that could provide detailed structural information about particular constituents directly from the extract before investing in isolation and purification (Jarosewski, 2004; Hostettmann, et al., 2001). If the structures of the extract constituents were known in advance, the isolation efforts could be focused only on novel and interesting compounds and this would increase the efficiency of the whole process. Since scarcity of the plant materials restricts large scale extraction and isolation procedures, novel techniques that could provide detailed structural information directly from the crude extract or less purified fractions of the crude extracts are essential for the phytochemical studies of rare or endangered medicinal plants.

However, with the outstanding developments in the areas of separation methods, spectrometric techniques, and sensitive bioassays, natural products research has gained momentum in recent years (Sticher, 2008). These novel approaches afford for a rapid identification and characterization of secondary metabolites without the necessity of isolation and purification while the detailed information about their metabolic profiles can be obtained with a minimal amount of material.

A detailed discussion on novel approaches in natural products chemistry is beyond the scope of this thesis, therefore only a brief discussion on several mass spectrometric approaches that have demonstrated great potential in fast and efficient exploration of secondary metabolites are presented here.

1.3 Mass spectrometry in natural product analysis

Recent advancements in mass spectrometry and novel separation techniques have remarkably widened their applications to the analysis of complex biomaterials and established a new paradigm in natural products research. With the availability of a number of modern sophisticated hyphenated techniques, such as gas chromatography–mass spectrometry (GC-MS), liquid chromatography–mass spectrometry (LC-MS), liquid chromatography with parallel nuclear magnetic resonance spectroscopy and mass spectrometry (LC-NMR-MS), and capillary electrophoresis-mass spectrometry (CE-MS), the pre-isolation analysis of crude extracts or fractions from different natural matrices, isolation, online detection and dereplication of natural products, studies on chemotaxonomy and biosynthesis, chemical finger-printing, quality control of herbal products, and metabolomic studies have now become much easier than ever before (Sarker and Nahar, 2012; Patel, et al., 2010).

In traditional mass spectrometric approaches in small molecule investigations, purified samples were subjected to high-energy (70 eV) electron ionization (EI) under high vacuum conditions (Hoffmann and Stroobant, 2007). Although this technique is applicable only for the analysis of thermally stable, low molecular weight volatile compounds, it produces consistent and fragment rich mass spectra which can be easily used for a mass spectral library search (Hocart, 2010). However, the low abundance or absence of molecular ion in the EI spectra in most of the situations appears to be problematic in the calculation of elemental composition. Therefore, a chemical ionization (CI) technique is employed to obtain molecular ion information though electron ionization is widely used in GC-MS setups (Kind and Fiehn, 2010).

In spite of the power in online separation and identification, the penetration of GC-MS into the field of natural products chemistry has been restricted by the polar and ionic character as well as low volatility of majority of the natural products (Colegate and Molyneux, 2007). Therefore, the development of electrospray ionization (ESI)

MS has marked a milestone in the analysis of natural products, as diverse classes of secondary metabolites are amenable to this method. As it can be directly coupled to a high performance liquid chromatography, ESI has turned in to be the ionization of choice for LC-MS in the identification and isolation of secondary metabolites from complex extracts (Cech and Enke, 2001; Xing, et al., 2007; Lim and Lord, 2002). The sample is sprayed into the ion source as a solution, where it is evaporated under atmospheric pressure in the presence of an electric field, charged ions so generated to be separated by the mass analyzer. In contrast to "hard" ionization techniques, ESI rarely generates fragments and the molecules are ionized by protonation, cationization, or deprotonation. ESI-MS experiments provide reliable evidence for the molecular weight of a compound and depending on the mass analyzer used, high accuracy in the determination of the molecular mass can be achieved from which the chemical formula and the number of double bonds, rings or heteroatoms can be inferred (Kind and Fiehn, 2010).

Atmospheric pressure chemical ionization (APCI) is another "soft ionization" technique and is considered as an ideal method of ionization for low- to medium-polar compounds. In contrast to the strong electric field desolvation in ESI, in APCI the conversion of the solvent into an aerosol is brought about by thermal energy. Continuous vaporization of the aerosol gives rise to gas phase molecules before ionization is initiated. This mixture of gas phase analytes, solvent and atomizing gas is then subjected to a discharge current produced from the corona discharge needle due to the application of high voltage. This leads to the generation of charged plasma through a combination of collisions and charge transfer reactions. Ultimately protonation or deprotonation reactions would take place and usually some degree of fragmentation that is useful for structural characterization could also occur (Byrdwell, 2001).

The "soft ionization" in the above mentioned atmospheric pressure ionization processes, specially in ESI, leads to little or no fragmentation, thus making it inconvenient for structural elucidation studies based on fragment ions. This issue was

addressed by the emergence of tandem mass spectrometry (MS/MS), in which an ion (called precursor ion) from the first stage of MS is selected and activated, to produce fragment ions, which are then analyzed in the second stage of MS. Collision-induced dissociation (CID), which consists in promoting the energy-controlled collision of a chemically inert gas, with the precursor ion is the most widely employed ion activation method. In order to optimize the MS/MS spectrum, the collision energy may be chosen, since low collision energy values promote soft fragmentation and produce few fragments, whereas high collision energy values prompt extensive fragmentation (Dias, et al., 2012). The resulted fragment ions allow individual components of complex mixtures to be characterized, thus indicating the potential of LC-MS/MS in natural products chemistry. This technique reduces the need for laborious separation procedures since structural elucidation of the compounds can often be performed directly from crude plant extracts (Pachuta, et al., 1988).

Although High Performance Liquid chromatography (HPLC) is an efficient analytical chromatographic technique for the separation of natural products in complex crude extracts, the recent introduction of ultra-HPLC (UHPLC) has provided new possibilities in liquid chromatography and demonstrated that it can advantageously replace existing HPLC methods for many applications, including quality control, profiling and fingerprinting, dereplication, and metabolomics (Eugster, et al., 2011). UHPLC results in a better separation while decreasing time and solvent consumption (Nováková, et al., 2006; Swartz, 2005) and UHPLC-MS/MS is considered as a high throughput analysis method in drug discovery compared to the normal HPLC-MS approaches (Chesnut and Salisbury, 2007).

Unlike the fragmentation by electron ionization in GC-MS, the fragmentation resulted from LC-MS/MS is less reproducible hence spectral library searches are problematic. Furthermore, the manual interpretation of CID spectra is cumbersome and requires expert knowledge, thus development of computational methods for fully automated analysis of such data was highly in demand (Rasche, et al., 2011).

The recently introduced automated method for annotating tandem MS data using a hypothetical fragmentation trees has opened a way to fast classification and identification of secondary metabolites. This approach resulted in hypothetical fragmentation trees in which nodes are annotated with molecular formulas of the fragments while the arcs represent fragmentation events. The automated comparison of fragmentation trees enables the automated analysis of large MS data sets for identifying unknown compounds and facilitates the understanding of secondary metabolism as well as the discovery of botanical therapeutics, biomarkers, signaling molecules etc. (Rasche, et al, 2012)

Another soft ionization technique, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) has drawn much attention over the recent years as a successful approach in natural products analysis. The sample is premixed with a UV absorbing matrix compound, and exposed to laser pulses which leads to desorption and ionization of the analytes at low pressure. MALDI is considerably tolerant to salts, does not frequently require pretreatment and allows for high throughput analysis, thus, reinforce a logical rationale to utilize in the studies of secondary metabolites (Cohen, et al., 2007). With the various developments in the field of MALDI, an atmospheric pressure interface, AP-MALDI, has also been presented allowing for much experimental flexibility, as it could be coupled with any mass spectrometer equipped with atmospheric pressure ionization. (Hoffmann and Stroobant, 2007)

Since its inception, MALDI-TOF has been considered to be a powerful tool in the analysis of large biomolecules like proteins, peptides and oligosaccharides. However, application of MALDI-MS towards small molecule analysis lagged behind due to saturation by matrix ions signals below 500 Da. in the spectrum. With the development of 1,8-bis(dimethylamino)naphthalene (DMAN), a super-base belonging to a class of compounds called “Proton sponges” as a novel matrix for the negative mode MALDI-MS analysis, the problem of low mass region interferences was

overcome and the potential of MALDI-TOF–MS technique for the analysis of low-molecular-weight compounds was well demonstrated (Shroff and Svatoš, 2009).

Furthermore, there are several reports on successful application of MALDI-TOF-MS in identification of secondary metabolites in intact microorganisms without any extraction procedures. This approach allowed to speed up the process of dereplication of secondary metabolites and to gain the information on the distribution of secondary metabolites within an organism (Grube, et al., 2007). Thus, MALDI profiling offers exceptionally high-throughput and a strategy for accelerating research on natural products.

Although only a few mass spectrometric approaches that facilitate rapid and efficient analysis of secondary metabolites have been summarized in this section, it is worthwhile to mention that there is an enormous number of other mass spectrometric techniques that have been successfully utilized in the field of natural products chemistry to reveal the mysteries of Nature.

1.4 Motivation for the thesis

The recent developments in mass spectrometry has tremendously expanded the understanding of the chemistry as well as the biological functions of secondary metabolites, which is crucial in diverse fields from the pharmaceutical industry to agriculture, where there is an immense number of potential applications. Therefore, the overall aim of this thesis is to develop and optimize mass spectrometric approaches for fast screening and discovery of natural products in a wide range of samples of biological origin, thus to broaden the horizons in metabolomics. This overall aim is achieved under three themes and a brief explanation of the rationale for each theme is given below.

Theme 1 - Development of novel MALDI matrices for metabolomic analysis

Since conventional MALDI matrices produce a forest of interfering peaks at low mass region in the spectrum, 1,8-bis(dimethylamino)naphthalene (DMAN) was developed as a novel matrix for the analysis of low molecular weight compounds (Shroff and Svatoš, 2009). DMAN is a super base belongs to a class of compounds called "Proton sponges". This name comes from the ability of these compounds to take up any available protons. The presence of two basic nitrogen centers in the molecule, having an orientation that allows the uptake of one proton to yield a stabilized $[N\text{----}H\text{----}N]^{+}$ intra molecular hydrogen bond is considered as a general feature of all proton sponges (Raab, et al., 2002). Beside these classical "proton sponges", a new class of proton sponges with exceptional basicities have also drawn the attention during the last two decades (Staab and Saupe, 1988). This group of compounds known as "Azahelicenes", have prospective applications in fields like optoelectronics, catalysis, sensors, etc. (Caronna, et al., 2012), however, remained rather unexplored. As some of the azahelicenes posses high proton affinities comparable to classical proton sponges (Roithová, et al., 2007), it would be rather intersting to investigate the effectiveness of this group of compounds as novel MALDI matrices in terms of achieved sensitivity, ion-lessness and suitability for the analysis of anions. Thereby the applicability of MALDI-TOF-MS could be enhanced for the analysis of low-molecular weight acidic metabolites in wide range of biological samples.

Theme 2 - Identification of bioactive secondary metabolites from medicinal plants in Sri Lanka

Sri Lanka has a high biodiversity among its flora which comprises over 3700 angiosperms and over 350 ferns, of which over 28% of flowering plants and 18% of ferns are endemic to the island. Plants and their products are the main components of the indigenous medicinal system in the country which has been practiced for over thousand years and is still popular among people, even though modern health care facilities are readily available in most part of the country. It is reported that over 1400 plants are used in indigenous medicine in Sri Lanka and herbal drugs have gained much attention and popularity in recent years because of their safety, efficacy and cost effectiveness (Wijesundera, 2004). Despite the rich biodiversity and the vital role of plants in traditional medicine, Sri Lankan flora has not yet been adequately studied phytochemically or pharmacologically. The requirement for large scale extractions as well as laborious isolation and purification methods which are highly technically demanding have hindered the chemical profiling of medicinal plants, hence the validation of their uses in traditional medicine. However, with the emergence of novel hyphenated techniques that facilitate online characterization of secondary metabolites in crude natural product extracts, together with the newly developed computer algorithms for the *de novo* identification of organic compounds based on their tandem mass spectra, invaluable information regarding the presence of bioactive metabolites could be obtained. Thereby, the extensive use of plants in Sri Lankan traditional medicine could be rationalized.

Theme 3 - Identification of sex dependent lipids in *Drosophila melanogaster*

The investigations on surface lipids of *Drosophila melanogaster* were initiated several decades ago, for the characterization of the components and later extended towards the determination of quantitative and qualitative differences between cuticular lipids in male and female flies (Jackson, et al., 1981). Thereafter, cuticular hydrocarbons in *D. melanogaster* have been subjected to intensive studies and several of these compounds have displayed a marked sexual dimorphism, thus pheromonal functions (Antony and Jallon, 1982; Jallon, 1984; Ferveur and Sureau, 1996; Ferveur, 2005; Foley, et al., 2007). Apart from the cuticular hydrocarbons, the understanding on other types of cuticular substances, especially fatty acids and their derivatives in *D. melanogaster* is not adequate, despite over a quarter century of investigations. Previous studies conducted on cuticular fatty acids have only revealed quantitative differences between fatty acid composition in male and female flies. No qualitative differences were observed however (Jackson, et al., 1981). To explore this sparsely known area and conduct in-depth studies on the cuticular non-hydrocarbon components that exist in minor quantities, advanced analytical approaches are desirable. Novel mass spectrometric techniques specially the UHPLC-APCI-MS, facilitate the detection and identification of even minor components in a complex surface lipid extract thus enabling the conduction of comprehensive studies on sex specific fatty acids and their derivatives. This could lead the field of insect cuticular chemistry to a new dimension.

To be concise, under the above mentioned themes, this thesis addresses different aspects in the field of natural products chemistry and demonstrates the capability of mass spectrometry to create a powerful platform which could move metabolomics to new heights.

1.5 Structure of the thesis

The thesis will be introduced in 8 chapters as follows:

Chapter 1 : General Introduction

Chapter 2 : Manuscript-I
Azahelicene superbases as MAILD matrices for acidic analytes

Chapter 3 : Manuscript-II
Inhibition of 5-lipoxygenase as anti-inflammatory mode of action of *Plectranthus zeylanicus* Benth and chemical characterization of ingredients by a mass spectrometric approach

Chapter 4 : Manuscript-III
Munronia pinnata (Wall.) Theob.: Unveiling phytochemistry and dual inhibition of 5-lipoxygenase and microsomal prostaglandin E_2 synthase (mPGES)-1

Chapter 5 : Manuscript-IV
Identification of female specific fatty acid derivatives in *Drosophila Melanogaster* surface lipid extracts

Chapter 6 : Discussion

Chapter 7 : Summary

Chapter 8 : References

OVERVIEW OF MANUSCRIPTS

Manuscript-I

Azahelicene superbases as MAILD matrices for acidic analytes

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Published in *ChemPlusChem* 2013 (78), 937-942.

In this manuscript, we attempted to explore the suitability of several azahelicenes as MALDI or MAILD matrices for the analysis of fatty acids and organic acids in wide range of samples and explain the differences in efficiency of individual azahelicenes using methods of theoretical chemistry. Out of the tested matrices, 1,14-diaza[5]helicene performed exceptionally well and resulted in clear deprotonated signals of the acid analytes without any matrix related peaks or alkali adducts formation. The higher gas phase proton affinity, higher pK_a , higher pK_b for deprotonation and the UV absorbance maximum in the frequency close to that of the lasers used, strongly favor a Matrix Assisted Ionization/Laser Desorption (MAILD) type of ionization, when this matrix is mixed with an acidic analyte. Development of this novel MAILD matrix for small molecule analysis demonstrates that MALDI-MS is no longer meant only for the high-molecular weight compound analysis.

Mayuri Napagoda designed and carried out all experiments on MALDI-MS, analyzed data and drafted the manuscript. Lubomír Rulíšek, Andrej Jančařík, Jiří Klívar, Michal Šámal, Irena G. Stará, Ivo Starý, Veronika Šolínová, Václav Kašička, involved in the synthesis of azahelicenes, calculation and measurement of the physiochemical parameters. Aleš Svatoš designed the study, analyzed the data and wrote the manuscript.

Manuscript-II

Inhibition of 5-lipoxygenase as anti-inflammatory mode of action of *Plectranthus zeylanicus* Benth and chemical characterization of ingredients by a mass spectrometric approach

Mayuri Napagoda, Jana Gerstmeier, Sandra Wesely, Sven Popella, Sybille Lorenz, Kerstin Scheubert, Aleš Svatoš and Oliver Werz
Published in Journal of Ethnopharmacology 2014 (151), 800-809

The herb *Plectranthus zeylanicus* is extensively used in traditional medicine in Sri Lanka and South India for the treatment of inflammatory disorders, however the pharmacological features of this plant are unexplored. This manuscript showed that *n*-hexane and dichloromethane extracts of *P. zeylanicus* potently suppress the activity of human 5-lipoxygenase (IC_{50} = 0.7-12 μg/ml) in cell-free and cell-based assays without significant radical scavenging activity or suppression of ROS formation. By means of UHPLC/ESI-MS and GC-MS analysis and also with the analysis of hypothetical fragmentation trees computed from the CID spectra, we identified coleone P, cinncassiol A / C, and callistric acid as uncommon constituents in the most active fractions of the separated extracts. In addition to the above compounds for which the knowledge regarding bioactivities are rare, the presence of compounds such as α- and β-amyrin with reported anti-inflammatory properties were also detected.

Mayuri Napagoda designed the experiments, collected the plants, carried out the experiments on extraction, fractionation and LC-MS measurements, analyzed the data and wrote the manuscript. Jana Gerstmeier, Sandra Wesely and Sven Popella conducted the bioassays. Sybille Lorenz carried out GC-MS measurements. Kerstin Scheubert computed the fragmentation trees. Aleš Svatoš and Oliver Werz designed the experiments, analyzed the data and wrote the manuscript.

Manuscript-III

Munronia pinnata (Wall.) Theob.: Unveiling phytochemistry and dual inhibition of 5-lipoxygenase & microsomal prostaglandin E_2 synthase-1

Mayuri Napagoda, Jana Gerstmeier, Andreas Koeberle, Sandra Wesely, Sven Popella, Sybille Lorenz, Kerstin Scheubert, Sebastian Boecker, Aleš Svatoš, and Oliver Werz

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Preparations from *Munronia pinnata* (Wall.) Theob is extensively used in traditional medicine in Sri Lanka for treating inflammatory conditions, however the pharmacological features or the phytochemistry of this plant are hardly explored in order to rationalize the reported ethnobotanical significance. Therefore in this manuscript, we explored the chemical profile as well as inhibition of 5-lipoxygenase (5-LO) and microsomal prostaglandin E_2 synthase (mPGES)-1 inhibition of this important medicinal plant. *n*-Hexane extract of *M. pinnata* efficiently suppressed 5-LO activity in stimulated human neutrophils (IC_{50} = 8.7 µg/ml) and potently inhibited isolated human recombinant 5-LO (IC_{50} = 0.48 µg/ml) and mPGES-1 (IC_{50} = 1.0 µg/ml). The phytochemistry of the plant was unveiled for the first time with the detection of phytosterols, fatty acids, sesquiterpenes and several other types of secondary metabolites. The chemical profiling was carried out solely by UHPLC/ESI-MS, UHPLC/APCI-MS and GC-MS analysis, without any extensive isolation and purification procedures.

Mayuri Napagoda designed the experiments, collected the plants, carried out the experiments on extraction, fractionation and LC-MS measurements, analyzed the data and wrote the manuscript. Jana Gerstmeier, Andreas Koeberle, Sandra Wesely, and Sven Popella conducted the bioassays. Sybille Lorenz carried out GC-MS measurements. Kerstin Scheubert computed the fragmentation trees under supervision of Sebastian Boecker. Aleš Svatoš and Oliver Werz designed the experiments, analyzed the data and wrote the manuscript.

Manuscript-IV

Identification of female specific fatty acid derivatives in *Drosophila melanogaster* surface lipid extracts

Mayuri Napagoda, Jerrit Weißflog, Sybille Lorenz and Aleš Svatoš

In preparation for the submission to ChemBioChem

Since the investigations carried out so far on sex dependent differences in composition of cuticular lipids in *Drosophila melanogaster* have been exclusively focused on cuticular hydrocarbons, the understanding on non-hydrocarbon components in the surface lipid extracts of male and female flies is rather primitive. In order explore this untouched area in the *D. melanogaster*'s cuticular chemistry, we have focused on identification of sex specific cuticular fatty acid and fatty acid derivatives and our achievements are summarized in this manuscript. A female specific 9-(3-methyl-5-pentylfuran-2-yl)nonanoic acid and its triglyceride was identified by UHPLC-APCI-MS and GC-MS methods and our finding challenges the decades-old concept of the absence of qualitative differences between cuticular fatty acid profiles in male and female flies.

Mayuri Napagoda designed and carried out experiments on preparation of flies for extraction, fractionation, LC-MS measurements, analyzed data and wrote the manuscript. Jerrit Weißflog carried out the synthesis of the fatty acids, NMR analysis and wrote the manuscript. Sybille Lorenz carried out GC-MS measurements. Aleš Svatoš designed the study, analyzed the data and wrote the manuscript.

Chapter 2

Manuscript-I

Azahelicene Superbases as MAILD Matrices for Acidic Analytes

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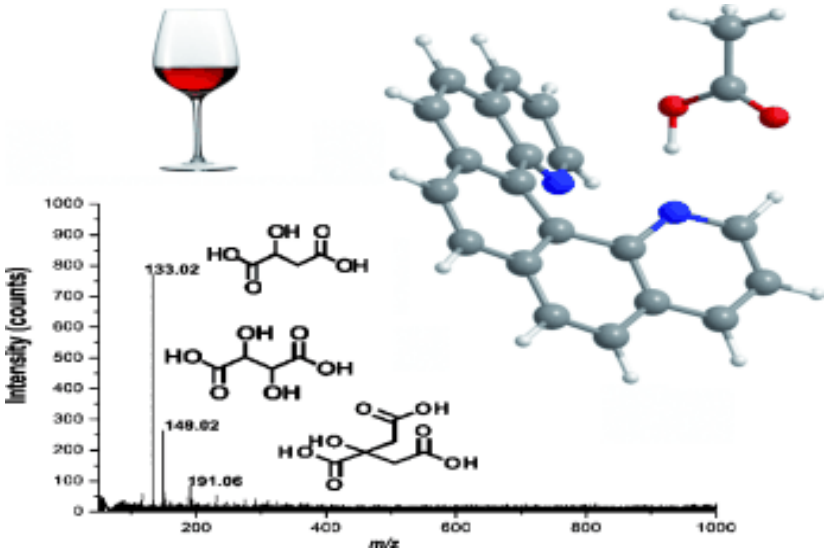

Abstract

A superbasic 1,14-diaza[5]helicene can serve as an efficient, ionless matrix for matrix-assisted ionization/laser desorption (MAILD) spectrometry. The matrix outperforms other bases by acting as a kinetically active proton sponge and is highly suitable for high-throughput metabolomics analysis. There is a correlation between the basicity (and proton-sponge character) of matrices and their efficacy in MAILD-MS.

Keywords: Helicenes, laser spectroscopy, mass spectrometry, matrix isolation, metabolomics

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SPECIAL ISSUE

Azahelicene Superbases as MAILD Matrices for Acidic Analytes**

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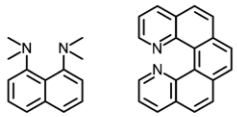
In memory of Detlef Schröder

Fast metabolite profiling, imaging and drug analysis is an indispensable tool nowadays in the discovery of biomarkers and disease diagnostic applications. Matrix-assisted laser desorption/ionisation (MALDI), developed in the late eighties, represents one high-throughput method. Initially used for the mass spectrometric analyses of large biomolecules,[1] for example, proteins, peptides and oligosaccharides, recently it has frequently been used to analyse small molecules.[2] However, conventional MALDI matrices (e.g., 2,5-dihydroxybenzoic or α-cyanohydroxycinnamic acids) produce a significant background of interfering peaks within the low-mass region; thus limiting the usefulness of MALDI-MS techniques in the analysis of low-molecular-mass analytes ($M_r < 500$ Da).[3]

To extend the MALDI-MS technique to include small-molecule analysis and to utilise this fast high-throughput screening technique for metabolomic studies,[4] we have recently developed a novel approach.[5] It is based on the usage of superbasic UV-absorbing matrices that do not undergo facile gas-phase deprotonation. Accordingly, no interfering anions are generated during the measurements. In contrast to the MALDI technique, ionisation is completed in a solution, if an acidic analyte is mixed with a super base; thus forming an ion pair. Such a phenomenon was explained by the Brønsted–Lowry acid–base theory and the new method was coined "matrix-assisted ionisation/laser desorption" (MAILD). Specifically, 1,8-bis(dimethylamino)naphthalene (DMAN; a "proton sponge")[6] was shown to obviate the problem of low-mass-region interference in MALDI-TOF/MS spectra of low-molecular-weight compounds at physiologically relevant concentrations.[5a,c] The same principle was applied to the positive-ion mode of MAILD by using 2-naphtholsulfonic acid.[5b] Recently, it was realised by us and others[7] that DMAN might desorb under experimental conditions (10^{-6} mbar) to form deposits in an ion source; these deposits might result in the appearance of interfering peaks in MS spectra. To eliminate this troublesome drawback, we have been searching for alternative MAILD matrices either by modifying the structure of DMAN or by exploring other types of organic superbases.



DMAN 1

To the best of our knowledge, azahelicenes[8] have never been tested as potential candidates for MALDI or MAILD matrices, despite the fact that some of them exhibit high proton affinities that are comparable to those of classical proton sponges.[9] Herein, we explore the potential of azahelicenes—the leading candidate is 1,14-diaza[5]helicene (**1**)[9c,10] —as MAILD matrices for the analysis of fatty acids and organic acids in a wide range of samples. The development of an efficient MAILD-MS analysis for small molecules may open up new directions in metabolomics research and diagnostic applications. We bring together our expertises in mass spectrometry, the synthesis of helical heteroaromatics, quantum chemical calculations and capillary electrophoresis not only to identify a new highly promising azahelicene matrix (**1**) for MAILD-MS and to explain differences in the efficiency of other azahelicenes, but also to shed light on the mechanisms underlying the MAILD technique.

Classical organic proton sponges, such as DMAN, are characterised both by a strong electrostatic repulsion between lone electron pairs at basic centres and by the hydrophobic shielding of these centres (as well as by the N–H$^+$–N hydrogen bond upon monoprotonation). Similar to DMAN, the conformational strain in **1** causes the lone electron pairs in nitrogen atoms to be in close proximity. Whereas protonation of DMAN (and deprotonation of DMANH$^+$) is a slow process (owing to hydrophobic shielding), helicene **1** is a kinetically active proton sponge (owing to a rapid exchange of protons between the donor and diaza[5]helicene base).[10b] It is worth noting that **1** should have a significantly lower vapour pressure under vacuum than that of DMAN because the scaffold of **1** consists of five condensed benzene/pyridine rings; thus minimising desorption.

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[**] *MAILD = matrix-assisted ionisation/laser desorption.*

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In initial experiments, a series of diverse azahelicene bases (**1**–**12**) were screened as potential MAILD matrices with respect to their sensitivity, for being ionless and general suitability for the analysis of anions at physiologically relevant concentrations. By using tartaric acid (TA) as an analyte and individual azahelicenes as a matrix, a very clear signal at *m/z* 149.02 $[M-H]^-$ of the monodeprotonated tartrate anion was observed only when **1** was used (Table 1 and Figure S1a in the Supporting Information). Interestingly, neither matrix-related peaks nor those corresponding to neutral losses of water or carbon dioxide were detected. In contrast, the MS spectra obtained with azahelicene matrices **2**–**12** either showed several peaks in the low-mass region (Table 1 and Figure S1c–l in the Supporting Information) or the analyte signal was very low (see Figure S1b in the Supporting Information).

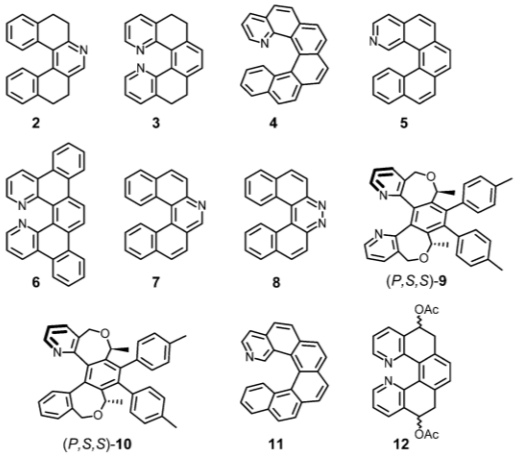
Table 1. Efficacy of tested matrices measured as M_{Eff} = analyte signal/total signal × 100 %.[a]

Matrix	TA[b]	SA[b]	ABA[b]	PA[b]	Average
1	100.0	100.0	100.0	100.0	**100.0**[d]
2	0.0	22.4[c]	47.82[c]	20.82[c]	22.76
3	88.06	100.0	38.49[c]	100.0	**81.64**[d]
4	40.02	16.01[c]	13.28[c]	13.60	20.72
5	42.78	6.76[c]	13.42[c]	4.40[c]	16.84
6	62.26	19.77	21.95	56.67	**55.72**[d]
7	63.18	15.86[c]	19.34[c]	23.58[c]	30.49
8	28.16[c]	19.05[c]	24.52[c]	6.61[c]	19.58
9	76.36	100.0	47.95[c]	100.0	**81.08**[d]
10	78.37	79.66	0.0	100.0[c]	**64.5**[d]
11	25.05	8.78	33.19	15.43	20.61
12	49.76	100.0	46.04	100.0	**73.95**[d]
DMAN	100.0	100.0	100.0	100.0	**100.0**[d]

[a] All peaks with intensities less than 1000 are ignored, if the intensity of the analyte peak is above 1000. [b] TA = tartaric acid, SA = stearic acid, ABA = abscisic acid, PA = palmitic acid. [c] Intensity of the analyte peak and/or base peak was less than 1000 and the calculation was done by considering the intensities of peaks, which were above $^1/_4$ of the intensity of the base peak. [d] Besides superior compound **1** (and DMAN as a reference), five additional azahelicenes, **3**, **6**, (*P,S,S*)-**9**, (*P,S,S*)-**10** and **12**, showed a reasonably good signal intensity without unwanted noise (indicated by bold values of average efficacy).

Next, the MAILD-MS spectra of additional acidic analytes, such as two fatty acids (PA and SA) and plant hormone (ABA), were measured under similar conditions by using the small library of azahelicene matrices **1**–**12** (Table 1). The results clearly showed that **1** was a superior matrix. In all cases, the efficiency of matrices, M_{Eff} (%) = 100(analyte signal/total signal), was calculated, and the average activity for all four acids tested was further used in the evaluation. However, these results were obtained only when an extensive MALDI target cleaning was applied (for details, see the Experimental Section).[11]

The initial experiments revealed the significant potential of **1** to serve as a new matrix for the MAILD-MS analysis of small molecules. Encouraged by these results, which demonstrated the superiority of **1** to DMAN, we embarked on using other acidic analytes to evaluate the versatility of this new matrix. Arachidic and ricinoleic acids provided clear deprotonated signals without any matrix-related peaks or the formation of alkali adducts (see Figure S2 in the Supporting Information). Elimination of water was registered only at ricinoleic acid and could possibly be explained by the allylic nature of the hydroxyl group. As in the DMAN matrix, we observed the maximum ion intensity with an equimolar ratio of the analyte and matrix **1**. Such an observation differed from the widely accepted concept of MALDI-MS, namely, that the excess of matrix relative to analyte has beneficial effects on the analysis (Figure 1a). To further explore the scope of the method, we turned our attention to biological samples of a natural origin, namely, fruit juices and wine. Again, clear deprotonated signals of organic acids in the samples were observed (Figure 1b–d and Figure S3 in the Supporting Information). The identity of these organic acids was further confirmed by accurate mass and tandem MS measurements on the AP-MALDI-Orbitrap system.



That **1** serves as a new efficient MAILD-MS matrix requires an explanation. Considering the kinetically active proton sponge **1** and juxtaposed hydrophobically shielded DMAN, we attempted to correlate gas- and condensed-phase basicities with the ability to ionise acidic analytes. Although the experimentally determined pK_a value of monoprotonated DMAN (12.2) is higher than that of monoprotonated **1** (8.75), the calculated gas-phase proton affinities are reversed (**1**: 1064 kJ mol^{-1}, DMAN: 1026 kJ mol^{-1}; Table 2 and Table S1 in the Supporting Information). As far as azahelicenes **2**–**12** are concerned, none exhibited such high basicity or proton affinity. In addition, azahelicenes **1**–**12** and DMAN are extremely weak C acids with pK_a values of around 40, as calculated by DFT (PBE) calculations (in ethanol).

We assume that mixing the proton sponge with the acidic analyte sample leads to the formation of a conjugated acid/base ion pair. Such an interaction of DMAN and **1** with acetic acid was calculated by DFT (PBE; Figure 2). Then, upon UV-laser irradiation at $\lambda = 337$ or 355 nm, which ideally overlaps with or is close to the absorption band of the original basic matrix, the ion pair absorbs light energy and evaporates from the target. For effective acetate anion liberation from the con-

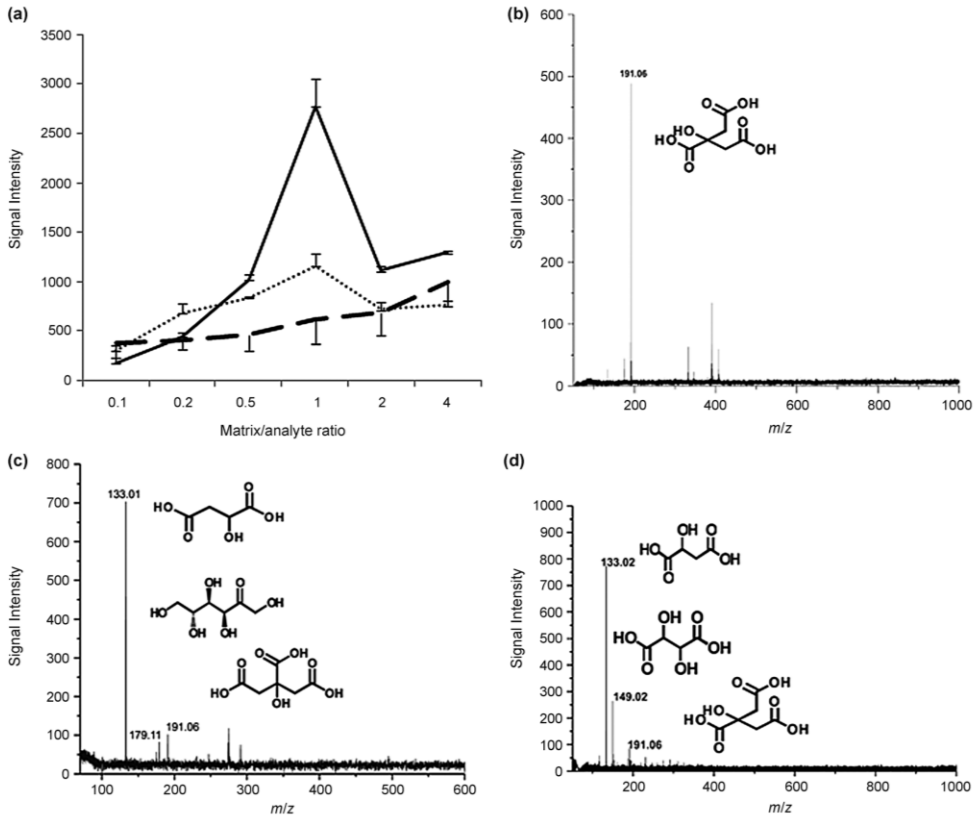


Figure 1. Dependence of ion signal intensities on analyte/matrix ratio and analysis of fruit samples. a) The signal intensity of the stearate anion was plotted for different matrices: **1** (——), (*P*,*S*,*S*)-**9** (••••) and 9-aminoacridine (- - - -); the amount of analyte was kept constant at 250 pmol, while the matrix concentration gradually increased (three replicates per concentration point); analysis of natural samples with matrix **1**: orange juice (b), apple juice (c) and white wine (d).

jugated ion pair, an elongated O–H bond is beneficial. Indeed, upon binding the free acetic acid to **1** and DMAN, the O–H bond length of 0.982 Å in acetic acid is substantially elongated in favour of the **1**·H^+–acetate conjugated ion pair (1.048 Å in **1**·HOAc vs. 1.030 Å in DMAN·HOAc). This is in accordance with the calculated dissociation enthalpy of the ion pair (B·HOAc → $BH^+ + AcO^-$), which is favourable for matrix **1** (400 kJ mol^{-1} for **1**·HOAc vs. 405 kJ mol^{-1} for DMAN·HOAc).

Upon considering the aforementioned physicochemical properties of **1** and DMAN, it comes as no surprise that **1** is a better MAILD matrix than DMAN because it effectively ionises/desorbs an acidic analyte without showing matrix-related peaks (Figure S1 a and S2 in the Supporting Information). In addition to **1**, azahelicenes **3**, **6** and **12** showed reasonably good signal intensities without unwanted noise (see bold values of the average efficacy in Table 1). They are structurally related to **1** and possess two nitrogen atoms in analogous positions; thus creating a claw for proton stabilisation (fuelled by cancelling the columbic repulsion between lone electron pairs in nitrogen atoms).[14] However, the partially hydrogenated scaffolds in **3** and **12** and the larger interplanar angle between the pyridine rings in **6**[15] allow the distance between the nitrogen atoms to be increased. As a result, they are weaker bases than **1** and, accordingly, their ionisation ability is lower. This relaxation effect is even more pronounced in (*P*,*S*,*S*)-**9**, which can be viewed as a double biaryl system that is free of helicene-type strain. Accordingly, the distance of the nitrogen atoms is doubled (4.9 Å in (*P*,*S*,*S*)-**9** vs. 2.65 Å in **1**) and, therefore, basicity is significantly lowered. Surprisingly, the ionising efficiency of this matrix is still high. Furthermore, the calculated value of $pK_a = 3.9$ (which is in excellent agreement with the experimental value of 3.83) of (*P*,*S*,*S*)-**9** does not correlate with the predicted trend for MAILD matrices (Figure 3).[16] Thus, we hypothesise that this compound behaves as a classical MALDI matrix. To support such reasoning, we compared the efficiency of matrices **1**, (*P*,*S*,*S*)-**9** and 9-aminoacridine (a classical MALDI matrix for negative-ion mode) by analysing SA under different analyte/matrix ratios (Figure 1 a). For matrix **1**, we measured

Table 2. Physicochemical properties of azahelicene bases **1**–**12** and DMAN.

Base B (matrix)	UV/Vis of B λ [nm] (ε [m^2 mol^{-1}])[a]	pK_a of BH$^+$ (exptl)[b]	PA of B (calcd) [kJ mol^{-1}][d]	pK_a of BH$^+$ (calcd)[e]
1	314 (17 344)	8.75[c]	1064	11.6
2	309 (5 853)	nd	nd	nd
3	318 (10 492)	6.84	1020	6.5
4	318 (18 845)	5.16[c]	1012	4.9
5	303 (22 715)	5.77[c]	992	6.2
6	292 (66 256)	6.43	1053	7.4
7	300 (25 973)	4.48	991	4.6
	377 (2 447)	–	–	–
	397 (3 097)	–	–	–
8	305 (13 224)	4.10	992	2.5
	324 (6133)	–	–	–
	396 (908)	–	–	–
	418 (979)	–	–	–
9	278 (24 206)	3.83	1007	3.9
10	249 (33 474)	3.79	1006	3.9
11	328 (23 633)	5.91	1003	6.1
12	nd	nd	1032	6.5
DMAN	341 (6 918)[f]	12.2	1026	9.7

[a] In chloroform: **1** (2.17×10^{-4} M), **3** (2.04×10^{-4} M), **4** (1.65×10^{-4} M), **5** (2.06×10^{-4} M), **6** (2.02×10^{-4} M), **9** (1.99×10^{-4} M), **10** (1.98×10^{-4} M), **11** (1.76×10^{-4} M); in acetonitrile: **2** (3.33×10^{-4} M), **7** (1.54×10^{-4} M), **8** (3.00×10^{-4} M); nd = not determined. [b] Measured by capillary electrophoresis in methanol (25 mM ion strength of the background electrolyte used and 25 °C). [c] Ref. [12]. [d] Gas-phase proton affinity (ΔG_{298K}) of B determined by DFT (PBE) calculations. [e] Related to the Gibbs energy of deprotonation (ΔG_{deprot}) of BH$^+$ in ethanol, as calculated by means of the COSMO method. [f] In chloroform, Ref. [13].

the highest signal at a 1:1 molar ratio, as expected for MAILD-type ionisation. For matrix (*P*,*S*,*S*)-**9**, however, the maximum at an equimolar ratio was insignificant, which was also true when 9-aminoacridine was used. Hence, matrix (*P*,*S*,*S*)-**9** is proposed to deprotonate an acid analyte in the gas phase and not in the condensed phase. Considering the same arguments, we might explain the behaviour of (*P*,*S*,*S*)-**10**, which has only one nitrogen basic centre and follows the matrix properties of (*P*,*S*,*S*)-**9**.

In summary, we demonstrated that **1** could serve as a new, efficient MAILD matrix, outperforming other bases such as DMAN, and was highly suitable for high-throughput metabolomics analysis. Matrix **1** acts as a kinetically active proton sponge by deprotonating acid analytes in the condensed phase. The highest efficiency in MAILD-MS analysis was obtained when **1** and the acid analyte were present in an equimolar ratio (no large excess of the matrix was needed). Furthermore, matrix **1** produced an insignificant background of interfering peaks within the low-mass region (< 500 Da). The superiority of **1** stemmed from the constructive interplay of its high basicity, ability to absorb energy of UV-light laser pulses, and resistance to fragmentation and desorption. Experimental and computational data led us to discover a rationale for the ionisation process: there is a correlation between the basicity (and proton-sponge character) of matrices and their efficacy in MAILD-MS. Further experiments with additional matrices [which may shed light on the mechanism of desorption/ionisation and explain how strongly bound MAILD ion pairs (dissociation enthalpy ca. 400 kJ mol^{-1}) dissociate in ion plumes] and resonance-enhanced multi-photon ionisation (REMPI), as well as infrared multi-photon dissociation (IRMPD), are currently under way.

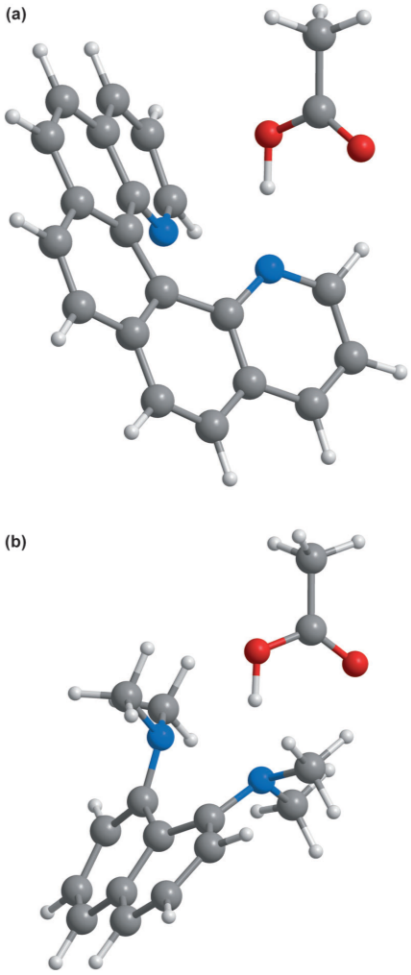


Figure 2. Calculated (DFT(PBE)/def2-SVP level) geometries of the complexes **1**·HOAc (a) and DMAN·HOAc (b) in ethanol.

Experimental Section

Materials

PA, SA and arachidic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA); PEG 600 sulfate and ricinoleic acid from TCI (Antwerp, Belgium); ABA from Alfa Aesar (Karlsruhe, Germany); TA was bought from Fluka (Buchs, Switzerland). HPLC grade solvents, ethanol, chloroform and acetone were purchased from Roth (Karlsruhe, Germany). Fruit juices and wine were purchased from a commercial market. The preparation of azahelicenes is described elsewhere (**1**,[10] **3**,[10b] **4**,[10b] **11**,[10b] **12**[10a]), is given in the Supporting Informa-

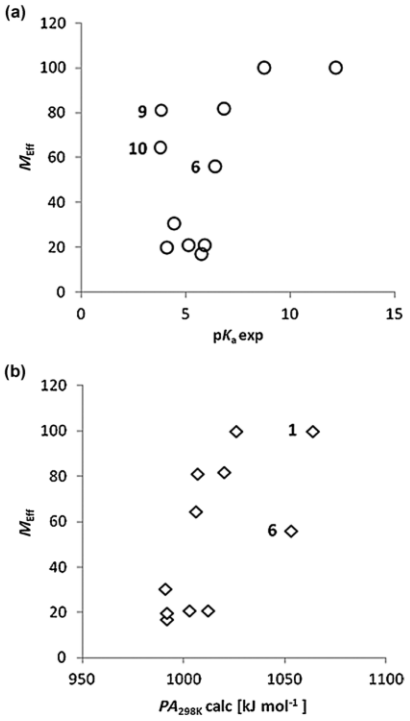


Figure 3. Correlation between experimental pK_a values (a) and calculated gas-phase protonation data (b) for azahelicenes **1**–**12** and their efficacy as matrices, M_{Eff}, values.

tion (**5** and the modified synthesis of **11**) or will be published separately (**2**, **6**,[15] **7**, **8**, (*P*,*S*,*S*)-**9**, (*P*,*S*,*S*)-**10**).

Sample preparation

Stock solutions of all azahelicene matrices, fatty acids and organic acids were prepared at a concentration of 10 nmol μL^{-1} in $CHCl_3$ or ethanol. Aliquots (1 μL) of the matrix and analyte solutions were mixed before MALDI measurements were performed. The resulting mixture (1 μL) was spotted on a circular metallic area with a diameter of 2.5 mm in the 96-well MALDI target plate. Fruit juices and wine were diluted 10-fold with distilled water and mixed with 1 nmol μL^{-1} of the helicene matrices and spotted on the MALDI target plate by following the same procedure.

Cleaning protocol for the MALDI stainless-steel target plate

The metallic MALDI plate (Waters) had to be extensively cleaned to remove traces of deposits from manufacturing or previous measurements, otherwise intense background signals are observed. The procedure was as follows: 1) The plate was initially washed with milli-Q water (50 mL). 2) The surface was rubbed with a metal polishing solution (Stahl fix classic, SC Johnson GmbH, Vienna, Austria) by using a laboratory brush. 3) It was rinsed with milli-Q water (100 mL). 4) It was sonicated with 30% formic acid (20 mL) for 15 min. 5) It was rinsed with milli-Q water (100 mL). 6) It was sonicated with 5 mg mL^{-1} of DMAN solution in ethanol (10 mL) for 15 min. 7) It was rinsed with methanol (50 mL, HPLC grade, Roth, Karlsruhe, Germany). 8) It was sonicated with CH_2Cl_2/methanol (2:1) for 15 min (20 mL). 9) It was rinsed with methanol (50 mL). 10) It was rinsed with hexane (50 mL, Roth HPLC grade, Karlsruhe, Germany). 11) It was heated in a laboratory oven (Roth, Germany) at 250 °C for 50 min.

Mass spectrometry

A MALDI micro MX mass spectrometer (Waters/Micromass, Manchester, UK) fitted with a nitrogen laser ($\lambda = 337$ nm, 4 ns laser pulse duration, repetition rate up to 20 Hz and 320 μJ per pulse) was used in reflectron and negative polarity modes for data acquisition. The instrument operated at 5×10^{-7} bar with a plate voltage of 5.2 kV, an acceleration voltage of −12 kV, and pulse and detector voltages of 1.95 and 2.10 kV, respectively. The laser frequency was set to 5 Hz and the energy was optimised for different analytes between 180 and 260 μJ per pulse. The extraction delay time was optimised to 150 ns. Polyethylene glycol (PEG) 600 sulfate was used to calibrate the mass spectrometer for an *m*/*z* range of 100–1000 Th in the negative-ion mode. MassLynx V4.0 software (Waters) was used for data acquisition and each spectrum was recorded with 10 laser pulses. The spectra obtained were smoothed, subtracted and centroided. The chemical identities of the organic acids detected in fruit juices and wine were confirmed by accurate mass measurements performed on an LTQ-Orbitrap XL instrument (Thermo Fisher, San Jose, CA) with an AP-MALDI source equipped with solid-state neodymium-doped yttrium–aluminium–garnet (Nd-YAG) laser (MassTech, Columbia, MD) and by using Xcalibur v.2.0 (Thermo) software for data acquisition.

Capillary electrophoresis

Measurements were performed in a bare fused silica capillary (total/effective length 305/195 mm, i.d./o.d. 50/375 μm) in a home-made apparatus equipped with a UV-absorption detector at $\lambda =$ 206 nm.[17] Data acquisition and handling were performed by using chromatography station Clarity (DataApex, Prague, CR) and the program Origin 6.1 (OriginLab Corp., Northampton, MA, USA), respectively. The separation voltage was +12 kV (anode at the injection end) and the electric current was in the range 11.7–14.5 μA. A more detailed description can be found in ref. [12].

Calculation procedures

Quantum chemical calculations were performed by using the TURBOMOLE 6.4 program and the RI-PBE+D3/def2-TZVP//RI-PBE+D3/def2-SVP model chemistry system. Solvation effects were taken into account by means of the COSMO-RS method[18] by using the COSMO*therm* program (BP86/def-TZVP parameter set). The Gibbs free energy was then calculated as the sum of the contributions in Equation (1):

$$G = E_{el} + G_{solv} + E_{ZPVE} - RT \ln(q_{trans} q_{rot} q_{vib}) \quad (1)$$

in which E_{el} is the in vacuo energy of the system, G_{solv} is the solvation free energy, E_{ZPVE} is the zero-point vibrational energy, and $-RT\ln(q_{trans}q_{rot}q_{vib})$ accounts for the entropic terms and the thermal correction to the enthalpy. The reported pK_a values were calculated by means of Equation (2):

$$pK_a = -\Delta G_{diss}/(RT \ln 10) \quad (2)$$

in which ΔG_{diss} is the free energy difference for the dissociation of the protonated form (acid) into the deprotonated form (base) and a proton. $\Delta G^{\circ}_{aq}(H^+) = -1118.2\ kJ\,mol^{-1}$ was used as the absolute solvation energy of the proton in ethanol, which included the correction of 7.9 $kJ\,mol^{-1}$ for processes in which the number of moles (Δn) changed. A full account of the computational details can be found in the Supporting Information.

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Keywords: helicenes · laser spectroscopy · mass spectrometry · matrix isolation · metabolomics

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Supporting Information

Azahelicene Superbases as MAILD Matrices for Acidic Analytes**

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Table S1: The primary computational data referring to the calculated values presented in the main article. E(PBE)/def2-TZVP is the molecular energy calculated at the DFT(RI-PBE+D3/def2-TZVP)

Suppl. Figure 1 Comparison of mass spectra for tartaric acid (250 pmol) with different azahelicene matrices (Compound **1-12**)

Suppl. Figure 2. MAILD spectra of series of acids (250 pmol) using 1,14-diaza[5]helicene (**1**) matrix

Suppl. Figure 3. MAILD spectra of beverages using 1,14-diaza[5]helicene (**1**) matrix

Experimental part for synthetic compounds with analytical data (9 pages)

Computation details (2 pages)

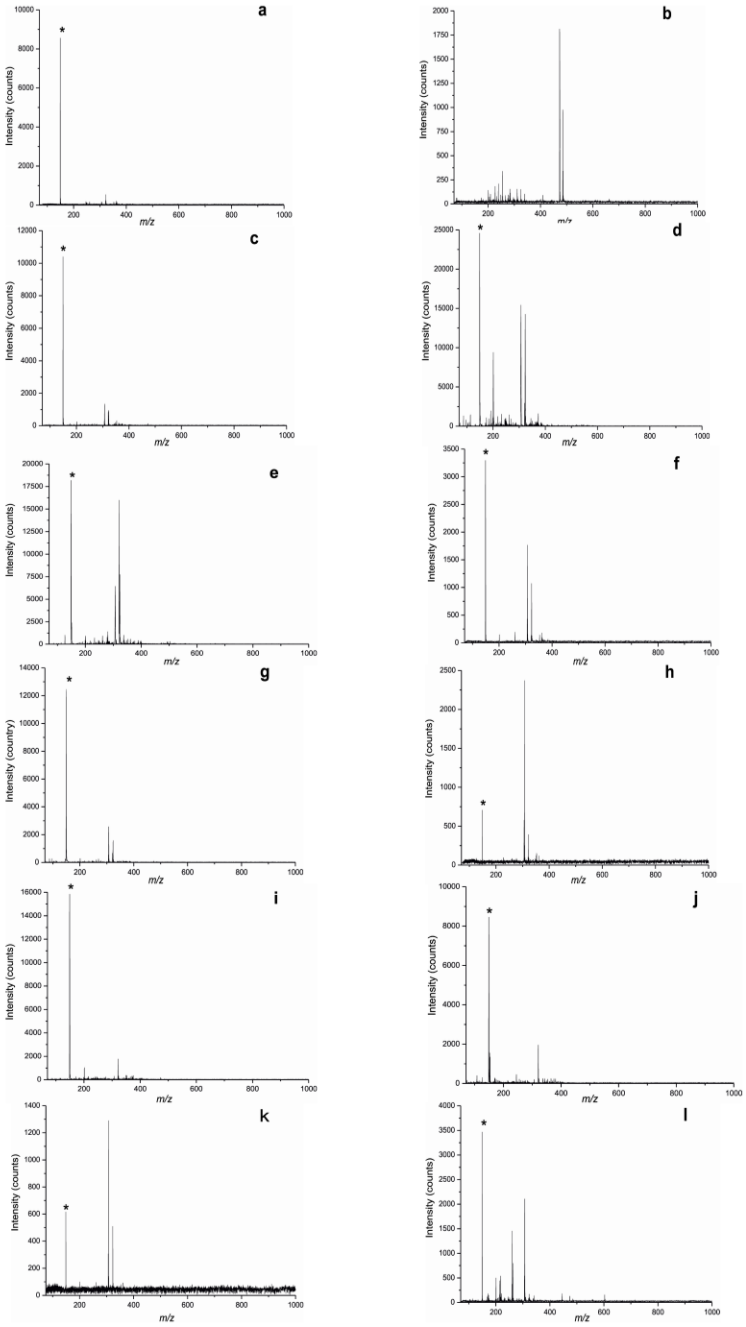
Table S1: The primary computational data referring to the calculated values presented in the main article. E(PBE)/def2-TZVP is the molecular energy calculated at the DFT(RI-PBE+D3/def2-TZVP) level; chemical potential is the $[E_{ZPVE} - RT \ln(q_{trans}\, q_{rot}\, q_{vib})]$ term, and the G_{solv}(COSMO-RS) is the solvation free energy calculated by the COSMO-RS method using the protocol described above. The first two terms sum up to gas-phase free energy - *G*tot(GP) - whereas the sum of the first three terms is *G*tot(solvent). From these values, the differences between the neutral and protonated species are calculated, e.g. proton affinity (PA), pK_a, and the proton affinity in solvent, $G_{protonation}$.

NEUTRAL	(ethanol)				(methanol)		
Compound	E(PBE)/def2-TZVP	Gsolv(COSMO-RS)	chem.pot.	Gtot(GP)	Gtot(ethanol)	Gsolv(COSMO-RS)	Gtot(methanol)
	[a.u.]	[kcal/mol]	[kJ/mol]	[a.u.]	[a.u.]	[kcal/mol]	[a.u.]
DMAN	-653.2049250	-9.87	634.5	-652.96325	-652.97897	-9.52	-652.97842
1	-878.1111302	-16.44	560.9	-877.89751	-877.92372	-16.46	-877.92374
3	-880.5052079	-16.57	673.5	-880.24869	-880.27509	-16.51	-880.27500
5	-862.0703518	-16	592.3	-861.84477	-861.87027	-15.97	-861.87022
7	-862.0739704	-15.38	592.7	-861.84824	-861.87275	-15.29	-861.87261
8	-878.0856101	-16.94	560.8	-877.87201	-877.89900	-17.02	-877.89913
4	-1015.5742583	-17.46	702.1	-1015.30686	-1015.33468	-17.34	-1015.33450
11	-1015.5724227	-18.47	702.8	-1015.30474	-1015.33418	-18.44	-1015.33413
6	-1185.1238209	-21.57	781.2	-1184.82628	-1184.86065	-21.55	-1184.86062
9	-1649.6030578	-27.59	1316.7	-1649.10156	-1649.14552	-27.29	-1649.14504
10	-1633.5617340	-27.36	1346.9	-1633.04873	-1633.09232	-26.94	-1633.09166
12	-1335.9623874	-22.98	848.5	-1335.63920	-1335.67582	-23.1	-1335.67601

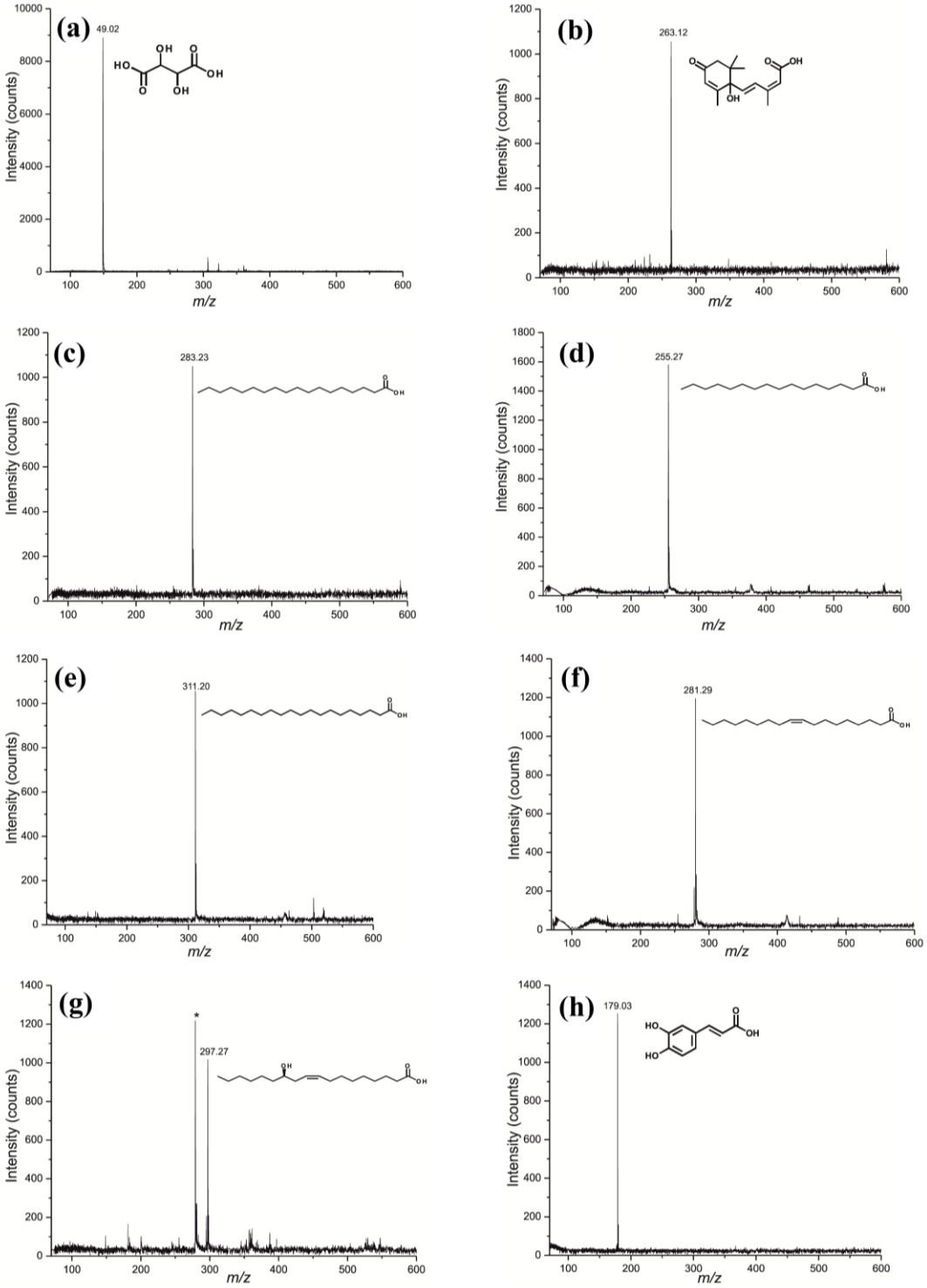
PROTONATED	(ethanol)				(methanol)		
	E(PBE)/def2-TZVP	Gsolv(COSMO-RS)	chem.pot.	Gtot(GP)	Gtot(ethanol)	Gsolv(COSMO-RS)	Gtot(methanol)
	[a.u.]	[kcal/mol]	[kJ/mol]	[a.u.]	[a.u.]	[kcal/mol]	[a.u.]
DMAN	-653.6061040	-45	661.5	-653.35415	-653.42587	-44.4	-653.42492
1	-878.5270629	-45.44	588.4	-878.30294	-878.37535	-44.8	-878.37433
3	-880.9071819	-49.09	708.6	-880.63730	-880.71552	-48.53	-880.71464
5	-862.4617982	-54.17	627.5	-862.22278	-862.30911	-54.17	-862.30911
7	-862.4646380	-52.13	627.5	-862.22562	-862.30870	-51.67	-862.30797
8	-878.4770731	-50.68	596.5	-878.24987	-878.33064	-50.22	-878.32991
4	-1015.9733251	-49.63	737.8	-1015.69230	-1015.77140	-49.09	-1015.77054
11	-1015.9676927	-54.53	738.1	-1015.68657	-1015.77348	-54.12	-1015.77282
6	-1185.5359800	-47.62	810.2	-1185.22739	-1185.30327	-46.9	-1185.30212
9	-1650.0000407	-59.55	1351.7	-1649.48519	-1649.58009	-58.87	-1649.57901
10	-1633.9578359	-59.6	1381.2	-1633.43177	-1633.52675	-58.85	-1633.52555
12	-1336.3683341	-52.69	882.7	-1336.03212	-1336.11609	-52.34	-1336.11553

CALCULATED Compound	PA(GP) [kJ/mol]	(ethanol) Gprotonation [kJ/mol]	(ethanol) pKa	(methanol) Gprotonation [kJ/mol]	(methanol) pKa
DMAN	1026.3	280.4	9.6	280.18	9.7
1	1064.4	283.4	11.8	282.74	11.6
3	1020.3	276.4	6.6	275.87	6.5
5	992.5	275.4	5.9	275.4	6.2
7	990.8	273.6	4.5	273.19	4.6
8	992.1	270.9	2.6	270.32	2.5
4	1012.0	274.0	4.9	273.62	4.9
11	1002.5	275.7	6.1	275.28	6.1
6	1053.1	277.7	7.6	277.04	7.4
9	1007.2	272.7	3.9	272.32	3.9
10	1005.7	272.6	3.8	272.26	3.9
12	1031.6	276.3	6.5	275.8	6.5

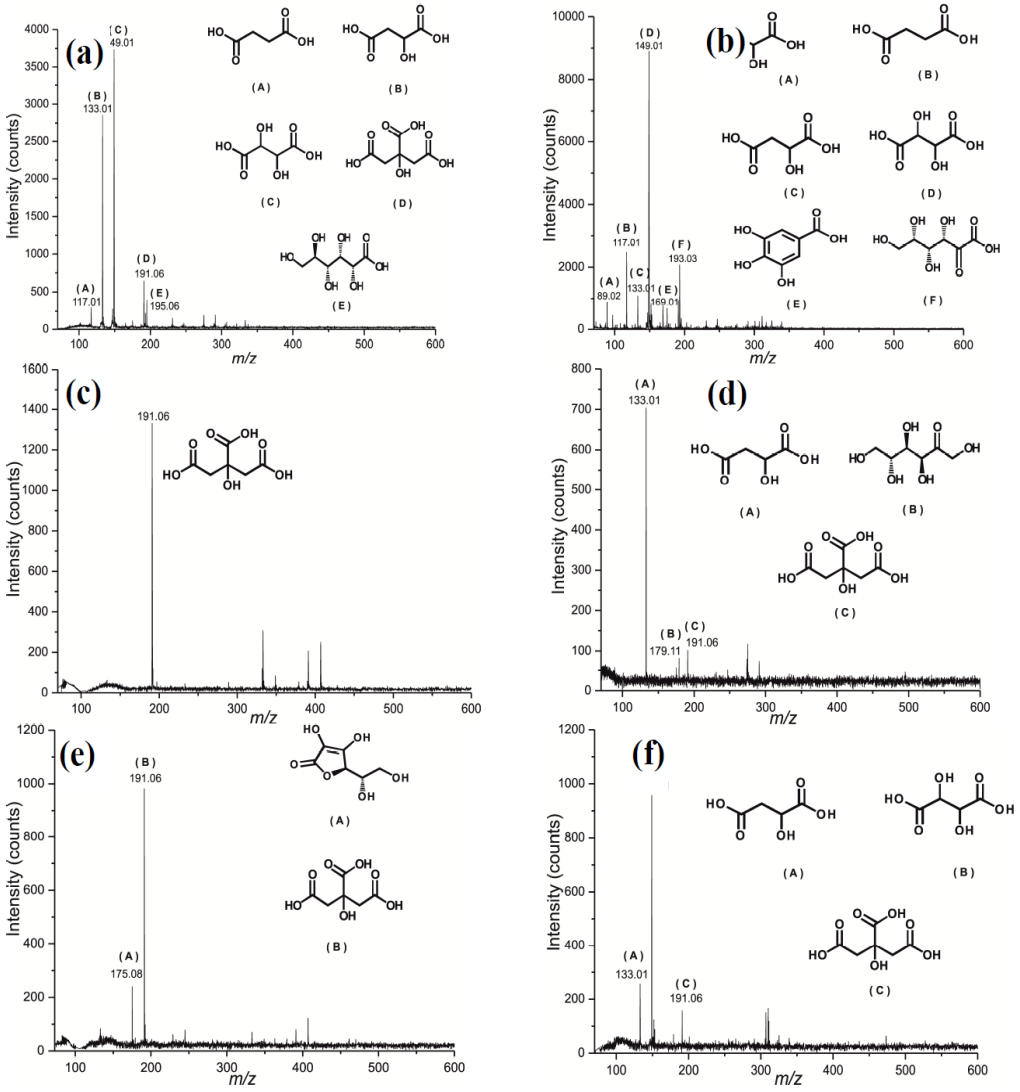
Supplementary Figures



Suppl. Figure 1 Comparison of mass spectra for tartaric acid (250 pmol) with different azahelicene matrices (Compound **1-12**) . (a)- **1**, (b)-**2**, (c)-**3**, (d)-**4**, (e)- **5**, (f)- **6**, (g)-**7**, (h)-**8**, (i)-**9**, (j)- **10**, (k)-**11**, (l)- **12. *** indicates the deprotonated anion of tartaric acid



Suppl. Figure 2. MAILD spectra of series of acids (250 pmol) using 1,14-diaza[5]helicene (**1**) matrix (**a**) tartaric acid, (**b**) abscisic acid, (**c**) stearic acid, (**d**) palmitic acid, (**e**) arachidic acid, (**f**) oleic acid, (**g**) ricinoleic acid, (**h**) caffeic acid; * Indicates a loss of H_2O.



Suppl. Figure 3. MAILD spectra of beverages using 1,14-diaza[5]helicene (**1**) matrix (**a**) wine-sample 1 (1/10 diluted with water), (**b**) wine-sample 2 (1/10 diluted), (**c**) orange juice (1/10 diluted), (**d**) apple juice (1/10 diluted), (**e**) pineapple juice (undiluted), (**f**) grape juice (1/10 diluted).

Experimental Section

General: Melting points are uncorrected. The ^{1}H NMR spectra were measured at 400.13 MHz and ^{13}C NMR spectra at 100.61 MHz in $CDCl_3$ in 5 mm PFG probe. For standardization of ^{1}H NMR spectra the internal signal of TMS (δ 0.0, $CDCl_3$) was used. In the case of ^{13}C spectra the residual signals of solvents (δ 77.00 for $CDCl_3$) were used. The chemical shifts are given in δ-scale, the coupling constants *J* are given in Hz. The IR spectra were measured in $CHCl_3$ on FT-IR spectrometer. The EI mass spectra were determined at an ionizing voltage of 70 eV, the *m/z* values are given along with their relative intensities (%). The standard 70 eV spectra were recorded in the positive ion mode. For exact mass measurement, the spectra were internally calibrated using perfluorotri-*n*-butylamine (Heptacosa). The ESI mass spectra were recorded using ZQ micromass mass spectrometer equipped with an ESCi multi-mode ion source and controlled by MassLynx software. Alternatively, the low resolution ESI mass spectra were recorded using a quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer and high resolution ESI mass spectra using a hybrid FT mass spectrometer combining a linear ion trap MS and the Orbitrap mass analyzer. As a mobile phase was used 80% methanol (flow rate 100 µl/min). Low and high resolution CI mass spectra were measured using an orthogonal acceleration time-of-flight mass spectrometer at an ionizing voltage of 70 eV, the m/z values are given with their relative intensities (%). The spectra were recorded in positive mode and the source temperature was 150 °C. Methane was present as a reagent gas in the CI source. For exact measurement the spectra were internally calibrated using Heptacosa or 2,4,6-tris(trifluoromethyl)-1,3,5-triazine (Metri). UV/Vis spectra were recorded with pure solvent (distilled MeCN or $CHCl_3$ for HPLC) as a baseline. The commercially available catalysts and reagent grade materials were used as received. The triethylamine was distilled from calcium hydride under argon and degassed by three freeze-pump-thaw cycles before use; the tetrahydrofuran was freshly distilled from sodium/benzophenone under nitrogen. The dichloromethane, methanol, and ethanol were used as purchased. TLC was performed on Silica gel 60 F_{254}-coated aluminium sheets (Merck) and spots were detected by the solution of $Ce(SO_4)_2$. 4 H_2O (1%) and $H_3P(Mo_3O_{10})_4$ (2%) in sulphuric acid (10%). The flash chromatography was performed on Silica gel 60 (0.040-0.063 mm, Fluka). Biotage Initiator EXP EU (300 W power) was used for reactions carried out in microwave oven. $Pd(PPh_3)_2Cl_2$ was purchased, CpCo(CO)(fum) was synthesised according to the literature procedure.[1] The starting materials **13**, **15**, and **21** were purchased, **19** was synthesized.[2]

Phenanthro[3,4-*h*]isoquinoline 5

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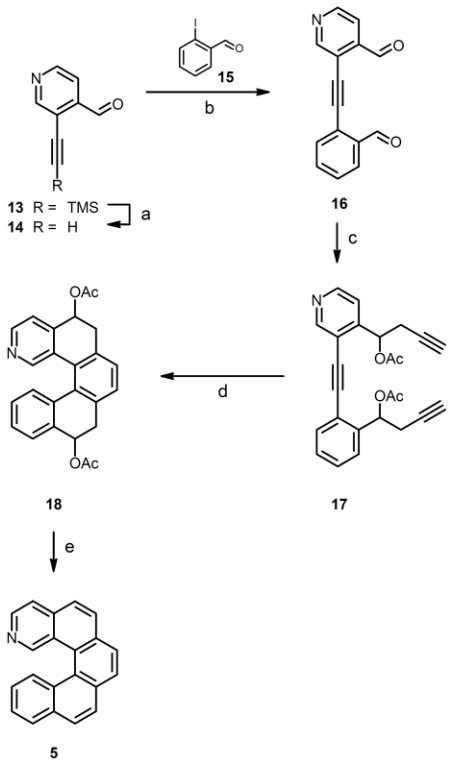
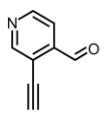
[1] A. Geny, N. Agenet, L. Iannazzo, M. Malacria, C. Aubert, V. Gandon, *Angew. Chem. Int. Ed.* **2009**, *48*, 1810–1813.

[2] M. Šámal, S. Chercheja, J. Vacek Chocholoušová, J. Vacek, J. Rybáček, D. Šaman, P. Fiedler, L. Bednárová, I. G. Stará, I. Starý, *To be submitted*.



(a) K_2CO_3 (5.0 equiv.), CH_2Cl_2-MeOH (10:3), rt, 5 min, 87%; (b) **15** (1.0 equiv.), $Pd(PPh_3)_2Cl_2$ (2 mol%), CuI (4 mol%), Et_3N-THF (4:3), 50 °C, 2 h, 77%; (c) propargyl bromide (3.0 equiv.), Zn dust (3.2 equiv.), THF, rt, 10 min, then Ac_2O (2.0 equiv.), Et_3N (2.0 equiv.), CH_2Cl_2, rt, 2 h, 87%; (d) CpCo(CO)(fum) (1.0 equiv.), 1-butyl-2,3-dimethylimidazolium tetrafluoroborate (5 mg/1mL of solvent), microwave reactor, 170 °C, 20 min, 82%; (e) trifluoromethanesulfonic acid (cat.), silica gel, CH_2Cl_2, 120 °C, 2 h, 53%.

3-Ethynylpyridine-4-carbaldehyde 14



Pyridine **13**[3] (1.01 g, 4.97 mmol) was dissolved in dichloromethane (10 mL) and methanol (3 mL) was added followed by potassium carbonate (24.85 g, 24.85 mmol, 5.0 equiv.). The suspension was stirred for 5 min and then neutralized by acetic acid (5 mL). The reaction mixture was quenched with brine (20 mL), the organic layer was separated and washed with water (20 mL), dried over anhydrous $MgSO_4$ and the solvents were evaporated *in vacuo*. The residue was purified by flash chromatography on silica gel (hexane-ethyl acetate 6:1) to afford the desired product **14** (570 mg, 87%) as an amorphous solid.

^{1}H NMR (400 MHz, $CDCl_3$): 3.59 (s, 1H), 7.69 (dd, J = 5.1, 0.8, 1H), 8.75 (d, J = 5.0, 1H), 8.92 (s, 1H), 10.50 (d, J = 0.7, 1H).

^{13}C NMR (101 MHz, $CDCl_3$): 76.76 (s), 87.14 (d), 119.35 (d), 120.06 (s), 141.54 (s), 150.21 (d), 155.28 (d), 190.42 (d).

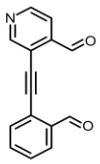
IR ($CHCl_3$): 3305 s, 3089 vw, 3050 w, 3031 w, 2855 w, 2744 w, 2114 w, 1712 vs, 1680 m, 1585 m, 1553 m, 1491 w, 1473 w, 1401 s, 1387 w, 1296 s, 1178 m, 1138 m, 1047 m, 1003 w, 933 w, 839 s, 660 m, sh, 639 s, 504 vw, 452 w, 400 w cm^{-1}.

EI MS: 131 ($M^{+\bullet}$, 90), 103 (98), 76 (100), 61 (8), 50 (47).

HR EI MS: calculated for C_8H_5ON 131.0371, found 131.0374.

[3] L. Melzig, C. B. Rauhut, N. Naredi-Rain, P. Knochel, *Chem. Eur. J.* **2011**, *17*, 5362-5372.

3-[(2-Formylphenyl)ethynyl]pyridine-4-carbaldehyde 16



A Schlenk flask was charged with 2-iodobenzaldehyde **15**[4] (1.01 g, 4.35 mmol), bis(triphenylphosphine)palladium dichloride (61 mg, 0.09 mmol, 2 mol%), copper iodide (33 mg, 0.18 mmol, 4 mol%), and purged with argon. The degassed triethylamine (12 mL) and tetrahydrofuran (6 mL) were injected and the mixture was warmed to 50 °C. Then **14** (570 mg, 4.35 mmol) in the degassed tetrahydrofuran (3 mL) was added dropwise. The reaction was stirred at 50 °C for 2 h. The organic solvents were evaporated *in vacuo* and the residue was purified by flash chromatography on silica gel (hexane-ethyl acetate 6:1) to afford **16** (792 mg, 77%) as slightly yellow crystals.

M.p. 130-131 °C (hexane-ethyl acetate).

^{1}H NMR (400 MHz, $CDCl_3$): 7.54-7.61 (m, 1H), 7.66 (td, *J* = 7.5, 7.5, 1.4, 1H), 7.74 (dd, *J* = 7.7, 0.8, 1H), 7.76 (d, *J* = 5.0, 1H), 7.99 (dd, *J* = 7.7, 1.0, 1H), 8.80 (d, *J* = 4.9, 1H), 9.03 (s, 1H), 10.57 (d, *J* = 0.5, 1H), 10.63 (s, 1H).

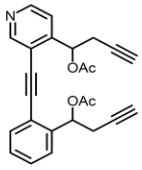
^{13}C NMR (101 MHz, $CDCl_3$): 88.61 (s), 94.77 (s), 120.21 (d), 124.34 (s), 129.07 (s), 129.07 (d), 130.06 (d), 134.01 (d), 134.01 (d), 134.67 (s), 141.06 (s), 150.17 (d), 154.75 (d), 190.43 (d), 190.74 (d).

IR ($CHCl_3$): 3029 w, 2847 w, 2743 w, 2217 vw, 1711 vs, sh, 1703 vs, 1594 m, 1583 w, 1567 w, 1552 w, 1486 m, 1451 w, 1403 m, 1392 w, sh, 1297 w, 1277 w, 1164 w, 1136 w, 1089 vw, 1048 w, 1001 vw, 866 vw, 837 m, 816 w, 706 vw, 555 w, 503 vw, 452 w cm^{-1}.

EI MS: 235 ($M^{+\cdot}$, 80), 207 (80), 178 (100), 151 (72), 126 (18), 98 (13), 87 (8), 75 (18), 63 (9), 51 (6).

HR EI MS: calculated for $C_{15}H_9O_2N$ 235.0633, found 235.0632.

1-[3-({2-[1-(Acetyloxy)but-3-yn-1-yl]phenyl}ethynyl)pyridin-4-yl]but-3-yn-1-yl acetate 17



In a Schlenk flask dried by a heat gun zinc dust (90 mg, 1.38 mmol, 3.2 equiv.) was placed and the flask was flushed with argon. The freshly distilled tetrahydrofuran (1.5 mL) was added followed by a propargyl bromide solution (80 wt % in toluene, 144 µL, 1.29 mmol, 3.0 equiv.). The resulting suspension was stirred at room temperature for 1 h. Then a solution of **16** (100 mg, 0.43 mmol) in tetrahydrofuran (1.5 mL) was added and the reaction mixture was stirred at room temperature for 10 min, then quenched with a solution of NH_4Cl (5 mL), the organic layer was separated, dried over anhydrous $MgSO_4$, and the solvent was evaporated in *vacuo*. The residue was dissolved in dichloromethane (3 mL), triethylamine (120 µL, 0.86 mmol, 2.0 equiv.), and acetic anhydride (81 µL, 0.86 mmol, 2.0 equiv.) added and the solution was stirred at room temperature for 2 h. The reaction mixture was quenched with brine (5 mL), the organic layer was separated, dried over anhydrous $MgSO_4$, and the solvents were evaporated *in vacuo*. The residue was purified by flash chromatography on silica gel (hexane-ethyl acetate 3:1 to 2:1) to afford **17** (148 mg, 87%) as an amorphous solid.

^{1}H NMR (400 MHz, $CDCl_3$, mixture of two diastereomers): 1.99-2.03 (m, 4H), 2.14 (s, 3H), 2.14 (s, 3H), 2.17 (s, 3H), 2.18 (s, 3H), 2.80-3.05 (m, 8H), 6.30 (m, 2H), 6.39 (m, 2H), 7.34 (td, *J* = 7.5, 7.5, 1.3, 2H), 7.40-7.44 (m, 2H), 7.43 (d, *J* = 5.8, 2H), 7.52 (d, *J* = 7.6, 2H), 7.59 (d, *J* = 7.6, 2H), 8.58 (d, *J* = 4.1, 2H), 8.80 (s, 2H).

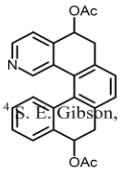
^{13}C NMR (101 MHz, $CDCl_3$, mixture of two diastereomers): 20.98 (q), 20.99 (q), 21.09 (q, 2C), 25.01 (t), 25.04 (t), 25.87 (t), 25.89 (t), 70.60 (d), 70.66 (d), 71.06 (d), 71.07 (d), 71.59 (d), 71.60 (d), 71.61 (d), 71.67 (d), 78.45 (s), 78.51 (s), 79.26 (s), 79.31 (s), 88.74 (s), 88.80 (s), 95.06 (s), 95.09 (s), 118.01 (s), 118.05 (s), 120.35 (d), 120.37 (d), 120.61 (s), 120.65 (s), 126.18 (d), 126.19 (d), 128.25 (d, 2C), 129.48 (d, 2C), 132.82 (d, 2C), 140.94 (s, 2C), 148.68 (s, 2C), 149.11 (d), 149.14 (d), 153.04 (d, 2C), 169.62 (s, 2C), 169.77 (s, 2C).

IR ($CHCl_3$): 3309 m, 3031 w, 2964 w, 2217 vw, 2125 vw, 1745 vs, 1587 w, 1556 w, 1493 w, 1403 m, 1374 m, 1237 vs, 1165 vw, 1045 m, sh, 1035 m, 936 w, 836 w, 653 m, 641 m, 609 vw cm^{-1}.

ESI MS: 422 ($[M+Na]^+$), 400 ($[M+H]^+$).

HR ESI MS: calcd for $C_{25}H_{22}O_4N$ 400.15433, found 400.15429.

5,6,9,10-Tetrahydrophenanthro[3,4-*h*]isoquinoline-5,10-diyl diacetate 18



A microwave vial was charged with **17** (94 mg, 0.23 mmol), CpCo(CO)(fum) (66 mg, 0.23 mmol, 1.0 equiv., fum = dimethylfumarate), 1-butyl-2,3-dimethylimidazolium tetrafluoroborate (20 mg, 5 mg / 1mL of solvent), a seal was closed, and vial was flushed with argon. The freshly distilled tetrahydrofuran (4 mL) was

[4] S. E. Gibson, N. Guillo, R. J. Middleton, A. Thuilliez; M. J. Tozer, *J. Chem. Soc., Perkin Trans. 1* **1997**, 447-456.

injected and the reaction mixture was heated to 170 °C in a microwave reactor for 20 min. Then the solvent was removed *in vacuo* and the residue was purified by flash chromatography on silica gel (hexane-ethyl acetate 3:1) to afford **18** (77 mg, 82%) as an amorphous solid.

^{1}H NMR (400 MHz, $CDCl_3$, mixture of diastereomers): 1.94 (s, 3H), 2.31 (s, 3H), 2.70-3.35 (m, 4H), 5.85-6.20 (m, 2H), 7.05-7.35 (m, 6H), 7.45-7.57 (m, 1H), 8.35-8.55 (m, 2H).

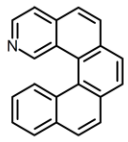
^{13}C NMR (151 MHz, $CDCl_3$, mixture of diastereomers): 21.29 (q), 21.46 (q), 34.97 (t), 35.20 (t), 69.57 (d, 2C), 117.25 (d), 127.93 (d, 2C), 128.32 (d), 128.53 (s), 128.91 (s), 129.01 (d), 129.34 (s), 129.75 (s), 129.84 (d, 2C), 132.13 (s), 134.08 (s), 134.58 (s), 145.27 (s), 147.92 (d), 150.29 (d), 170.59 (s), 171.07 (s).

IR ($CHCl_3$): 3065 vw, 2960 w, 1734 vs, 1599 w, 1591 w, 1571 vw, 1557 vw, 1490 w, 1468 vw, sh, 1446 w, sh, 1436 w, 1425 w, 1408 m, 1374 m, 1244 vs, 1129 vw, 1048 m, 1040 m, 1024 m, 991 w, 955 w, 696 vw, 614 w cm^{-1}.

ESI MS: 400 ($[M+H]^+$).

HR ESI MS: calcd for $C_{25}H_{22}O_4N$ 400.15433, found 400.15420.

Phenanthro[3,4-*h*]isoquinoline 5



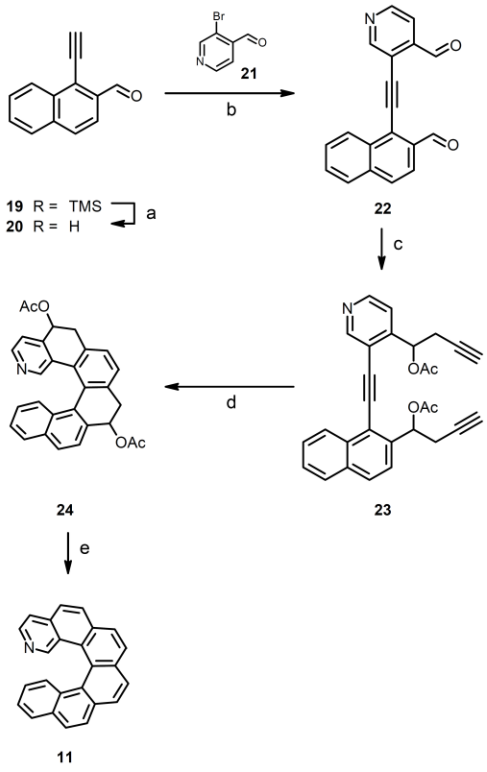
To a solution of **18** (43 mg, 0.11 mmol) in dichloromethane (2 mL) silica gel (2 g) and trifluoromethanesulfonic acid (2 drops, cat.) were added. The solvent was evaporated *in vacuo*, the residue was heated to 120 °C while stirred for 2 h. After cooling to the room temperature, the solid was directly loaded to a silica gel column and purified by the flash chromatography (hexane-ethyl acetate 6:1 with 5% of triethylamine) to afford **5** (16 mg, 53%) as an amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum was in agreement with published data.[5]

M.p. 208-209 °C (hexane-ethyl acetate).

^{13}C NMR (101 MHz, $CDCl_3$): 120.37 (d), 125.62 (d), 125.66 (d), 126.21 (s), 126.25 (d), 126.42 (s), 126.97 (d), 127.29 (d), 127.83 (d), 128.23 (d), 128.30 (d), 128.30 (s), 128.48 (d), 131.13 (d), 131.19 (s), 132.61 (s), 132.79 (s), 132.87 (s), 135.74 (s), 143.90 (d), 151.49 (d)

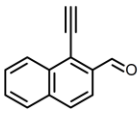
Benzo[5,6]phenanthro[3,4-*h*]isoquinoline 11

[5]S. Abbate, C. Bazzini, T. Caronna, F. Fontana, C. Gambarotti, F. Gangemi, G. Longhi, A. Mele, I. N. Sora, W. Panzeri, *Tetrahedron* **2006**, *62*, 139-148.



(a) K_2CO_3 (5.0 equiv.), EtOH, rt, 1 h, 98%; (b) **21** (1.2 equiv.), $Pd(PPh_3)_2Cl_2$ (5 mol%), CuI (10 mol%), Et_3N-THF (3:1), 45 °C, 2 h, 91%; (c) propargyl bromide (4.0 equiv.), Zn dust (8.0 equiv.), THF, rt, 1 h, then Ac_2O (4.0 equiv.), Et_3N (4.0 equiv.), rt, 2 h, 65%; (d) CpCo(CO)(fum) (0.8 equiv.),1-butyl-2,3-dimethylimidazolium tetrafluoro-borate (5 mg/1mL of solvent), microwave reactor, 170 °C, 20 min, 63%; (e) trifluoro-methanesulfonic acid (cat.), silica gel, CH_2Cl_2, 120 °C, 2 h, 68%.

1-Ethynylnaphthalene-2-carbaldehyde 20



1-[(Trimethylsilyl)ethynyl]naphthalene-2-carbaldehyde **19**[6] (2.00 g, 7.92 mmol) was dissolved in ethanol (10 mL). To this solution potassium carbonate (1.64 g, 11.88 mmol, 1.5 equiv.) was added and the mixture was stirred at room temperature for 1 h. After this period, the reaction was quenched with a solution of NH_4Cl (10 mL), extracted with diethyl ether (2 x10 mL), and the combined organic layers were dried over anhydrous $MgSO_4$. The solvents were evaporated under reduced pressure and the residue was purified by flash chromatography on silica gel (hexane-diethyl ether 20:1) to afford aldehyde **20** (1.40 g, 98%) as a white solid.

M.p. 138-138.5 °C (hexane-diethyl ether).

^{1}H NMR (400 MHz, $CDCl_3$): 3.92 (s, 1H), 7.61-7.76 (m, 2H), 7.88-7.92 (m, 1H), 7.91 (d, J = 9.0, 1H), 7.98 (d, J = 8.6, 1H), 8.53-8.57 (m, 1H), 10.79 (d, J = 0.8, 1H).

^{13}C NMR (101 MHz, $CDCl_3$): 77.23 (s), 90.23 (d), 121.95 (d), 126.08 (s), 127.17 (d), 127.96 (d), 128.54 (d), 129.50 (d), 129.55 (d), 133.47 (s), 135.35 (s), 135.70 (s), 191.94 (d).

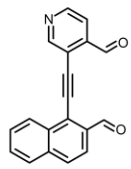
IR ($CHCl_3$): 3302 s, 3064 w, 2866 w, 2851 w, 2756 vw, 2729 vw, 2110 vw, 2101 vw, 1697 vs, 1681 vs, 1620 w, 1592 m, 1568 w, 1508 vw, 1461 m, 1433 m, 1402 w, 1376 m, 1335 m, 1269 w, 1231 s, 1156 vw, 1145 w, 1027 w, 958 w, 915 w, 872 w, 823 s, 706 vw, 660 s, 640 m, sh, 629 m, 571 m, 436 w cm^{-1}.

[6] M. Šámal, S. Chercheja, J. Vacek Chocholoušová, J. Vacek, J. Rybáček, I. G. Stará, I. Starý, *To be submitted*.

CI MS: 181 ([M+H]$^+$), 153 (6).

HR CI MS: calcd for $C_{13}H_9O$ 181.0653, found 181.0658.

3-[(2-Formylnaphthalen-1-yl)ethynyl]pyridine-4-carbaldehyde 22



A Schlenk flask was charged with 3-bromo-4-pyridine-carboxaldehyde **21** (430 mg, 2.30 mmol), bis(triphenylphosphine)palladium dichloride (80 mg, 0.12 mmol, 5 mol%), copper iodide (44 mg, 0.23 mmol, 10 mol%), and purged with argon. The degassed triethylamine (12 mL) and tetrahydrofuran (2 mL) were injected, and the mixture was warmed to 45 °C. Then aldehyde **20** (500 mg, 2.75 mmol, 1.2 equiv.) in the degassed tetrahydrofuran (2 mL) was added dropwise. The reaction was stirred at 45 °C for 2 h. The organic solvents were evaporated *in vacuo* and the residue was purified by flash chromatography on silica gel (hexane-ethyl acetate 5:1) to afford dialdehyde **22** (601 mg, 91%) as slightly yellow crystals.

M.p. 163-164 °C (hexane-ethyl acetate).

^{1}H NMR (400 MHz, $CDCl_3$): 7.72-7.77 (m, 2H), 7.81 (dd, *J* = 5.0, 0.7, 1H), 7.94-7.97 (m, 1H), 7.99 (d, *J* = 8.7, 1H), 8.04 (d, *J* = 8.6, 1H), 8.58-8.63 (m, 1H), 8.86 (d, *J* = 5.0, 1H), 9.16 (s, 1H), 10.67 (s, 1H), 10.88 (d, *J* = 0.7, 1H).

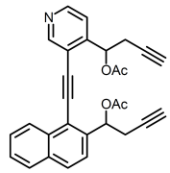
^{13}C NMR (101 MHz, $CDCl_3$): 92.19 (s), 94.83 (s), 119.59 (s), 120.89 (d), 122.62 (d), 125.04 (s), 126.93 (d), 128.42 (d), 128.80 (d), 129.72 (d), 130.36 (d), 133.17 (s), 135.12 (s), 135.76 (s), 140.84 (s), 150.43 (d), 154.88 (d), 189.99 (d), 191.15 (d).

IR ($CHCl_3$): 3090 vw, sh, 3063 w, 2864 w, sh, 2847 m, 2760 vw, sh, 2738 w, 2202 w, 1712 vs, 1698 vs, 1683 s, 1618 w, 1591 m, 1583 w, sh, 1567 w, 1552 w, 1508 vw, 1485 w, 1474 w, sh, 1459 m, 1433 m, 1405 s, 1400 m, sh, 1383 m, 1334 m, 1271 w, 1257 m, 1160 w, 1147 w, 1136 w, 1071 vw, 1045 vw, sh, 1027 w, 999 vw, 958 w, 931 vw, sh, 918 w, 871 w, 837 m, 824 s, 700 w, 656 m, 639 w, 570 w, 555 w, 546 w, 495 w, 451 w, 433 w cm^{-1}.

EI MS: 285 ($M^{+\bullet}$, 18), 257 (10), 228 (18), 200 (11), 167 (17), 149 (100), 111 (7), 104 (11), 97 (12), 83 (15), 71 (22), 57 (40), 44 (43).

HR EI MS: calculated for $C_{19}H_{11}O_2N$ 285.0790, found 285.0794.

1-[3-({2-[1-(Acetyloxy)but-3-yn-1-yl]naphthalen-1-yl}ethynyl)pyridin-4-yl]but-3-yn-1-yl acetate 23



In a Schlenk flask dried by a heat gun zinc dust (658 mg, 10.08 mmol, 8.0 equiv.) was placed and the flask was flushed with argon. The freshly distilled tetrahydrofuran (6 mL) was added followed by a propargyl bromide solution (80 wt % in toluene, 560 µL, 5.04 mmol, 4.0 equiv.). The resulting suspension was stirred at room temperature for 1 h. Then a solution of dialdehyde **22** (350 mg, 1.23 mmol) in tetrahydrofuran (4 mL) was added, and the reaction mixture was stirred at room temperature for 1 h. Then triethylamine (702 µL, 5.04 mmol, 4.0 equiv.), and acetic anhydride (477 µL, 5.04 mmol, 4.0 equiv.) were added, and the solution was stirred at room temperature for additional 2 h. The reaction mixture was quenched with brine (20 mL), the organic layer was separated, dried over anhydrous $MgSO_4$, and the solvents were evaporated *in vacuo*. The residue was purified by flash chromatography on silica gel (hexane-ethyl acetate 3:1) to afford triyne **23** (356 mg, 65%) as an amorphous solid.

^{1}H NMR (400 MHz, $CDCl_3$, mixture of two diastereomers): 2.02 (t, *J* = 2.5, 2H), 2.05 (dd, *J* = 4.6, 2.1, 2H), 2.16 (s, 6H), 2.19 (s, 3H), 2.19 (s, 3H), 2.90-3.10 (m, 8H), 6.44 (m, 2H), 6.68 (m, 2H), 7.46 (d, *J* = 5.1, 2H), 7.56 (t, *J* = 7.4, 2H), 7.64 (d, *J* = 8.5, 2H), 7.65 (t, *J* = 7.7, 2H), 7.87 (d, *J* = 8.1, 2H), 7.91 (d, *J* = 8.6, 2H), 8.47 (d, *J* = 8.4, 2H), 8.62 (d, *J* = 4.9, 2H), 8.95 (s, 2H).

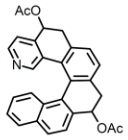
^{13}C NMR (101 MHz, $CDCl_3$, mixture of two diastereomers): 21.01 (q, 2C), 21.14 (q, 2C), 25.26 (t), 25.33 (t), 25.97 (t), 26.01 (t), 70.85 (d), 70.91 (d), 71.19 (d, 2C), 71.61 (d), 71.66 (d), 72.16 (d), 72.25 (d), 78.50 (s), 78.59 (s), 79.26 (s), 79.31 (s), 93.00 (s), 93.03 (s), 94.32 (s), 94.39 (s), 118.01 (s, 2C), 118.16 (s), 118.20 (s), 120.49 (d), 120.53 (d), 123.10 (d), 123.12 (d), 126.40 (d), 126.43 (d), 127.03 (d, 2C), 127.76 (d), 127.77 (d), 128.40 (d, 2C), 129.86 (d, 2C), 132.84 (s, 2C), 133.09 (s), 133.11 (s), 140.06 (s), 140.09 (s), 148.63 (s, 2C), 149.27 (d, 2C), 153.22 (d), 153.25 (d), 169.65 (s), 169.81 (s).

IR ($CHCl_3$): 3309 s, 3062 w, 2984 w, 2920 w, 2857 vw, 2205 vw, 2125 vw, 1745 vs, 1621 vw, 1587 w, 1569 vw, 1556 w, 1509 w, 1487 w, 1464 vw, 1431 w, 1406 s, 1373 s, 1235 vs, 1072 m, sh, 1047 s, 1033 s, 951 vw, 867 w, 822 m, 653 m, 641 m, 609 w, 568 w, 552 vw, 538 vw cm^{-1}.

ESI MS: 472 ([M+Na]$^+$), 450 ([M+H]$^+$).

HR ESI MS: calcd for $C_{29}H_{23}O_4NNa$ 472.15193, found 472.15202.

5,6,9,10-Tetrahydrobenzo[5,6]phenanthro[3,4-*h*]isoquinoline-5,10-diyl diacetate 24



A microwave vial was charged with triyne **23** (350 mg, 0.78 mmol), CpCo(CO)(fum) (184 mg, 0.62 mmol, 0.8 equiv., fum = dimethylfumarate), and 1-butyl-2,3-dimethylimidazolium tetrafluoro-borate (100 mg, 5 mg / 1mL of solvent), a seal was closed, and vial was flushed with argon. The freshly distilled tetrahydrofuran (18 mL) was injected and the reaction mixture was heated to 170 °C in a microwave reactor for 20 min. Then the solvent was removed *in vacuo* and the residue was purified by flash chromatography on silica gel (hexane-ethyl acetate 2:1) to afford diacetate **24** (220 mg, 63%) as an amorphous solid.

^{1}H NMR (400 MHz, $CDCl_3$, mixture of four diastereomers): 1.92 (s, 3H), 1.93 (s, 3H), 2.10 (s, 3H), 2.12 (s, 3H), 2.34 (s, 3H), 2.35 (s, 6H), 2.36 (s, 3H), 2.82-2.95 (m, 4H), 3.05-3.15 (m, 4H), 3.61-3.23 (m, 2H), 3.26-3.39 (m, 6H), 5.98 (t, *J* = 2.8, 1H), 6.00 (t, *J* = 2.7, 1H), 6.11-6.15 (m, 2H), 6.15-6.27 (m, 4H), 6.80-6.86 (m, 2H), 6.92-6.99 (m, 2H), 7.15-7.24 (m, 4H), 7.28-7.35 (m, 4H), 7.36-7.44 (m, 8H), 7.52 (d, *J* = 8.8, 1H), 7.55 (d, *J* = 10.1, 2H), 7.60 (d, *J* = 8.5, 2H), 7.61 (t, *J* = 4.2, 1H), 7.65 (d, *J* = 7.8, 2H), 7.69 (d, *J* = 4.0, 2H), 7.71-7.76 (m, 6H), 7.82 (d, *J* = 8.2, 1H), 7.83 (d, *J* = 8.3, 1H), 7.87 (d, *J* = 8.5, 2H), 8.10-8.17(m, 4H).

^{13}C NMR (101 MHz, $CDCl_3$, mixture of four diastereomers): 21.15 (q), 21.19 (q), 21.29 (q, 2C), 21.35 (q, 2C), 21.44 (q, 2C), 34.24 (t), 34.25 (t), 34.92 (t), 34.96 (t), 35.26 (t), 35.33 (t), 36.34 (t), 36.42 (t), 68.81 (d), 68.87 (d), 69.67 (d), 69.73 (d), 70.04 (d), 70.12 (d), 71.50 (d), 71.56 (d), 117.03 (d, 2C), 120.70 (d), 120.74 (d), 123.11 (d), 123.15 (d), 125.21 (d), 125.23 (d), 125.32 (d), 125.33 (d), 125.66 (d, 2C), 125.78 (d), 125.83 (d), 125.89 (d), 126.26 (d), 126.98 (d), 127.03 (d), 127.74 (d), 128.05 (d), 128.07 (d), 128.36 (d), 128.39 (d, 2C), 128.42 (d, 3C), 128.46 (d, 2C), 128.60 (s), 128.61 (s), 128.64 (s), 128.66 (s), 128.70 (d), 128.84 (d), 128.99 (d), 129.03 (d), 129.09 (d), 129.21 (d), 129.85 (d), 130.33 (s), 130.34 (s), 130.47 (s, 2C), 130.49 (s), 132.05 (s), 132.30 (s), 132.75 (s), 132.98 (s), 133.21 (s), 133.25 (s), 133.37 (s), 133.54 (s), 133.58 (s), 134.59 (s, 2C), 134.64 (s, 2C), 135.11 (s, 2C), 135.16 (s, 2C), 135.67 (s), 135.71 (s, 2C), 136.47 (s, 2C), 136.66 (s), 139.59 (s, 2C), 139.69 (s, 2C), 143.87 (s, 2C), 143.98 (s), 147.55 (d, 2C), 147.66 (d, 2C), 148.33 (d), 148.41 (d), 148.63 (s), 148.78 (d), 148.89 (d), 170.53 (s), 170.57 (s, 3C), 170.75 (s, 2C), 171.13 (s), 177.16 (s).

IR ($CHCl_3$): 3060 w, 2959 m, 2925 w, 1735 s, 1620 vw, 1600 w, 1581 w, 1555 vw, 1510 w, 1457 vw, 1436 w, 1373 m, 1243 s, 1073 w, 1049 m, 1022 m, 993 w, 868 w, 838 w, 822 m, 632 vw, 611 w, 590 vw, 568 w, 519 vw cm^{-1}.

ESI MS: 472 ($[M+Na]^+$), 450 ($[M+H]^+$).

HR ESI MS: calcd for $C_{29}H_{24}O_4N$ 450.1700, found 450.1698.

Benzo[5,6]phenanthro[3,4-*h*]isoquinoline 11

To a solution of diacetate **24** (220 mg, 0.49 mmol) in dichloromethane (4 mL) silica gel (6 g) and trifluoromethanesulfonic acid (5 drops, cat.) were added. The solvent was evaporated *in vacuo*, the residue was heated to 120 °C while stirred for 2 h. After cooling to the room temperature, the solid was directly loaded to a silica gel column and purified by flash chromatography (hexane-ethyl acetate-triethylamine 10:2:1) to afford **11** (110 mg, 68%) as a yellow solid. NMR spectra were in agreement with published data.[7]

[7] J. Míšek, F. Teplý, I. G. Stará, M. Tichý, D. Šaman, I. Císařová, P. Vojtíšek, I. Starý, *Angew. Chem. Int. Ed.* **2008**, *47*, 3188-3191.

Computational Details

Quantum chemical calculations were performed using the TURBOMOLE 6.4 program. All calculations were carried out at the DFT level, using the Perdew-Burke-Ernzerhof (PBE) functional[i] which included Grimme's empirical dispersion correction[ii] in its version 3 (PBE+D3). DFT (PBE) calculations were expedited by expanding the Coulomb integrals in an auxiliary basis set, the resolution-of-identity (RI-J) approximation.[iii] For the geometry optimization, the def2-SVP basis set was employed on all of the atoms,[iv] whereas the def2-TZVP basis set was used for the final single-point calculations to obtain presumably more accurate molecular energies (i.e. RI-PBE+D3/def2-TZVP// RI-PBE+D3/def2-SVP 'model chemistry').[v]

Solvation effects were taken into account by using the COSMO-RS (conductor-like screening model for real solvents) method[vi] and employing TURBOMOLE 6.4 for the COSMO calculation.[vii] The recommended protocol involves the optimization of molecular geometry both *in vacuo* and in the implicit solvent (in our case, ε_r = 24.8 was used, corresponding to ethanol) followed by the single-point calculation with $\varepsilon_r = \infty$ (ideal screening). These data are processed by the COSMO*therm*[viii] program (COSMO-RS calculation). Becke-Perdew (B-P) functional[ix] was used for the *in-vacuo*, ε_r = 24.8, and the $\varepsilon_r = \infty$ calculations (i.e., parts of the composite COSMO-RS protocol) along with the def-TZVP basis set was used as it corresponds to available COSMO-RS database.

The Gibbs free energy was then calculated as the sum of the following contributions:

$$G = E_{el} + G_{solv} + E_{ZPVE} - RT \ln(q_{trans}\, q_{rot}\, q_{vib}), \qquad (1)$$

where E_{el} is the *in vacuo* energy of the system (at the RI-PBE+D3/def2-TZVP//RI-PBE+D3/def2-SVP level), G_{solv} is the solvation free energy (calculated using the RI-BP/def-TZVP(COSMO-RS, $\varepsilon = 1$, $\varepsilon = \infty$) method as described above), E_{ZPVE} is the zero-point vibrational energy, and $-RT \ln(q_{trans}\, q_{rot}\, q_{vib})$ accounts for the entropic terms, and the thermal correction to the enthalpy and is obtained from a frequency calculation using the same method and software as for the geometry optimization at the RI-PBE+D3/def2-SVP level, 298 K, and 1 atm using the ideal-gas approximation.[x]

The reported pK_a values were calculated according to the equation:

$$pK_a = -\Delta G_{diss}/(RT \ln 10) \qquad (2)$$

where ΔG_{diss} is the free energy difference for the dissociation of the protonated form (acid) into the deprotonated form (base) and a proton. We used the value of $\Delta G^0_{aq}(H^+)$= -1118.2 kJ.mol^{-1} as the absolute solvation energy of the proton in ethanol. This value also includes the correction of 7.9 kJ.mol^{-1} (corresponding to the difference between the concentration of the ideal gas at 298K and 1 atm and its 1mol.l^{-1} concentration) which is relevant for processes in which the number of moles (Δn) changes.

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All geometries, in MOLDEN .xyz format (Cartesian coordinates)
The structures are labelled according to the labeling in the
main text.
Molecule 1(H+) 1 protonated
35
c -3.44778 -0.01131 2.64951
c -2.05033 0.03617 2.77069
n -1.24958 0.03775 1.70349
c -1.68284 -0.00475 0.41118
c -3.11548 -0.05658 0.22564
c -3.96641 -0.05850 1.36280
c -3.67888 -0.10435 -1.08230
c -2.83688 -0.09538 -2.15743
c -1.40938 -0.04008 -2.01099
c -0.74680 0.00467 -0.72603
c -0.68320 -0.03106 -3.23660
c 0.68199 0.02255 -3.23578
c 1.40531 0.06348 -2.00917
c 0.73969 0.05412 -0.72504
c 2.83330 0.11098 -2.15368
c 3.67299 0.14523 -1.07724
c 3.10653 0.13280 0.23015
c 1.67330 0.09081 0.41349
c 3.95474 0.16206 1.36895
c 3.43306 0.15168 2.65532
c 2.03525 0.11328 2.77452
n 1.23725 0.08540 1.70554
h 3.24034 0.11687 -3.17543
h 4.76564 0.18101 -1.19692
h -4.77119 -0.14566 -1.20340
h -3.24153 -0.12973 -3.17957
h -5.05442 -0.09762 1.19909
h -4.08976 -0.01168 3.54084
h -1.55332 0.07389 3.75383
h -0.00402 0.06397 1.82229
h 4.07315 0.17339 3.54772
h 5.04328 0.19253 1.20694
h -1.24409 -0.06582 -4.18190
h 1.24499 0.03225 -4.18042
h 1.53544 0.10552 3.75690
Molecule 1
34
c -3.14769 0.53444 2.62531
c -1.80614 0.98779 2.52094
n -1.04519 0.80107 1.45314
c -1.56003 0.15204 0.37682
c -2.95024 -0.21889 0.32594
c -3.72326 -0.05086 1.50744
c -3.52831 -0.66694 -0.90588
c -2.78000 -0.65515 -2.05869
c -1.38388 -0.31851 -2.03945
c -0.72018 -0.06360 -0.79644
c -0.65972 -0.19663 -3.26761
c 0.66007 0.19646 -3.26755
c 1.38409 0.31839 -2.03931
c 0.72027 0.06353 -0.79637
c 2.78022 0.65502 -2.05843
c 3.52842 0.66682 -0.90554
c 2.95021 0.21883 0.32623

c 1.55999 -0.15208 0.37699
c 3.72312 0.05080 1.50781
c 3.14743 -0.53448 2.62563
c 1.80588 -0.98781 2.52113
n 1.04504 -0.80109 1.45325
h 3.24079 0.90510 -3.02695
h 4.59180 0.95321 -0.92359
h -4.59168 -0.95338 -0.92402
h -3.24048 -0.90522 -3.02726
h -4.77975 -0.36341 1.50581
h -3.71820 0.68608 3.55396
h -1.34739 1.54252 3.36093
h 3.71786 -0.68613 3.55434
h 4.77961 0.36334 1.50628
h -1.19347 -0.37216 -4.21461
h 1.19390 0.37195 -4.21450
h 1.34704 -1.54252 3.36109
Molecule 3(H+)
39
c -3.16445 0.64682 2.60928
c -1.87481 1.15218 2.52051
n -1.14424 0.91801 1.40489
c -1.56146 0.18070 0.33645
c -2.89434 -0.31089 0.37337
c -3.67306 -0.07959 1.51544
c -3.39226 -1.02579 -0.85054
c -2.91375 -0.26982 -2.09537
c -1.41060 -0.10431 -2.08454
c -0.70839 0.01199 -0.84823
c -0.68902 -0.03815 -3.28686
c 0.69717 0.13575 -3.27679
c 1.41885 0.17535 -2.06900
c 0.72546 0.06346 -0.83567
c 2.92849 0.26748 -2.07897
c 3.45818 0.96805 -0.82448
c 2.90755 0.28086 0.39759
c 1.55048 -0.14542 0.38921
c 3.67018 0.00049 1.54356
c 3.08528 -0.68452 2.61659
c 1.73775 -1.06958 2.50508
n 0.98937 -0.80834 1.42547
h 3.26386 0.79095 -2.99619
h 4.56548 0.97303 -0.80488
h -4.49499 -1.11691 -0.82477
h -3.40435 0.72942 -2.13871
h -4.70085 -0.47289 1.55061
h -3.76563 0.83024 3.50979
h -1.39129 1.74473 3.30931
h 3.66260 -0.92653 3.52094
h 4.72606 0.31109 1.58498
h -1.22711 -0.11356 -4.24413
h 1.24419 0.20800 -4.22981
h 1.24347 -1.62201 3.32358
h -2.97788 -2.05919 -0.86251
h -3.22052 -0.80408 -3.01574
h 3.35259 -0.76165 -2.13600
h 3.13556 2.03424 -0.83141
h -0.17550 1.25676 1.37952
Molecule 3
38
c -3.06586 0.66618 2.59468

c -1.76978 1.18581 2.42726
n -1.02419 0.97326 1.33901
c -1.53281 0.21014 0.35077
c -2.85699 -0.30496 0.38573
c -3.61862 -0.07498 1.54128
c -3.37749 -1.01512 -0.83839
c -2.91436 -0.25772 -2.09105
c -1.40635 -0.11761 -2.10512
c -0.71327 -0.00452 -0.87109
c -0.68379 -0.07784 -3.30967
c 0.70628 0.08884 -3.30217
c 1.41631 0.12411 -2.09007
c 0.71049 0.00624 -0.86372
c 2.92404 0.26463 -2.05974
c 3.37371 1.01768 -0.79961
c 2.84084 0.30278 0.41641
c 1.51739 -0.21277 0.36575
c 3.59057 0.06859 1.57884
c 3.02738 -0.67711 2.62345
c 1.73339 -1.19673 2.44057
n 0.99898 -0.98009 1.34558
h 3.26969 0.78246 -2.97754
h 4.47842 1.10164 -0.75134
h -4.48267 -1.09866 -0.80199
h -3.38778 0.75142 -2.10107
h -4.64623 -0.46841 1.60632
h -3.63534 0.86357 3.51540
h -1.31787 1.81585 3.21530
h 3.58743 -0.87796 3.54920
h 4.61721 0.46238 1.65621
h -1.22438 -0.15215 -4.26694
h 1.25670 0.16676 -4.25353
h 1.27376 -1.83023 3.22134
h -2.97316 -2.05259 -0.87282
h -3.25059 -0.77216 -3.01425
h 3.39788 -0.74432 -2.06838
h 2.96920 2.05507 -0.83445
Molecule 5(H+)
36
c -3.26100 0.64655 2.57682
n -1.96260 1.08698 2.52703
c -1.14331 0.86503 1.48144
c -1.58042 0.15032 0.35078
c -2.98719 -0.19770 0.30704
c -3.78417 0.01532 1.46995
c -3.54945 -0.67553 -0.90975
c -2.77915 -0.66464 -2.05516
c -1.38707 -0.32391 -2.04485
c -0.71521 -0.08872 -0.79677
c -0.66905 -0.20077 -3.27248
c 0.64911 0.20138 -3.25793
c 1.37390 0.31158 -2.02972
c 0.72638 0.01340 -0.78334
c 2.75544 0.69445 -2.04368
c 3.49496 0.71721 -0.88507
c 2.94822 0.21200 0.34130
c 1.57294 -0.21473 0.38507
c 3.77563 0.03997 1.48774
c 3.30366 -0.60901 2.62103
c 1.99121 -1.14257 2.62337
c 1.14995 -0.94987 1.53220

h 3.21086 0.97701 -3.00469
h 4.54413 1.04955 -0.89891
h -4.60829 -0.97004 -0.94051
h -3.23599 -0.92652 -3.02235
h -4.83856 -0.29532 1.47533
h -3.82367 0.85523 3.49610
h -1.59737 1.62500 3.32011
h -0.13427 1.28941 1.55421
h 3.95915 -0.75129 3.49287
h 4.81573 0.39730 1.44313
h -1.19692 -0.37613 -4.22155
h 1.18241 0.39572 -4.20094
h 1.64342 -1.73746 3.48138
h 0.15623 -1.41988 1.53819
Molecule 5
35
c -3.21087 0.63090 2.56863
n -1.94796 1.12653 2.57641
c -1.18411 0.89857 1.51531
c -1.58986 0.16415 0.35671
c -2.97579 -0.21022 0.30453
c -3.76928 -0.00084 1.46541
c -3.53248 -0.70134 -0.92177
c -2.77215 -0.68983 -2.06610
c -1.38176 -0.32933 -2.04540
c -0.72738 -0.06586 -0.79665
c -0.65865 -0.20198 -3.27182
c 0.65520 0.21009 -3.26429
c 1.37480 0.32468 -2.03387
c 0.72029 0.04130 -0.78957
c 2.76263 0.69085 -2.04851
c 3.51024 0.69510 -0.89644
c 2.95681 0.19390 0.32803
c 1.57106 -0.19860 0.37452
c 3.78328 0.00144 1.47153
c 3.29993 -0.63382 2.60762
c 1.97302 -1.12791 2.61549
c 1.13496 -0.91561 1.52661
h 3.21522 0.97301 -3.01190
h 4.56550 1.00911 -0.91146
h -4.58927 -1.00838 -0.94510
h -3.21953 -0.96178 -3.03518
h -4.82675 -0.30794 1.46512
h -3.80497 0.79275 3.48543
h -0.17811 1.34947 1.53715
h 3.95249 -0.78530 3.48071
h 4.83067 0.33823 1.42241
h -1.18766 -0.38466 -4.21999
h 1.19019 0.40283 -4.20715
h 1.60034 -1.69127 3.48419
h 0.11947 -1.33151 1.54845
Molecule 7(H+)
36
c -3.37692 0.63984 2.56987
c -2.04524 1.11743 2.61921
c -1.17344 0.90108 1.55692
c -1.59342 0.20527 0.38893
c -2.98146 -0.17667 0.29992
c -3.83920 0.01485 1.41862
c -3.51137 -0.66312 -0.93937
c -2.74467 -0.66892 -2.08139

c -1.36934 -0.30743 -1.99999
c -0.71956 -0.02825 -0.75676
n -0.63531 -0.22077 -3.16705
c 0.64468 0.17096 -3.20941
c 1.37647 0.32703 -2.01999
c 0.72919 0.04129 -0.76078
c 2.76249 0.70206 -2.07745
c 3.51772 0.70464 -0.93321
c 2.98142 0.19602 0.29942
c 1.59524 -0.20203 0.38005
c 3.83029 0.00139 1.42180
c 3.36595 -0.64334 2.56294
c 2.03925 -1.13781 2.60268
c 1.17281 -0.91574 1.53919
h 3.20008 0.97761 -3.04846
h 4.57176 1.01912 -0.96115
h -4.56841 -0.96543 -0.98384
h -3.17752 -0.94597 -3.05497
h -4.88840 -0.30790 1.34095
h -4.05121 0.79609 3.42473
h -1.69507 1.67386 3.50121
h -0.15285 1.29965 1.61580
h 4.03778 -0.80136 3.41970
h 4.87638 0.33672 1.35791
h -1.12463 -0.41537 -4.04756
h 1.09870 0.30696 -4.20162
h 1.69239 -1.70888 3.47641
h 0.15507 -1.32352 1.58412
Molecule 7
35
c -3.39042 0.61964 2.57202
c -2.06304 1.10998 2.62581
c -1.19208 0.90412 1.56130
c -1.59709 0.20034 0.39007
c -2.98069 -0.19221 0.29704
c -3.84017 -0.00709 1.41704
c -3.49705 -0.68450 -0.94825
c -2.71785 -0.66665 -2.07873
c -1.33609 -0.28168 -2.02091
c -0.71594 -0.02174 -0.75249
n -0.68079 -0.17165 -3.22118
c 0.57556 0.21153 -3.19515
c 1.35836 0.32561 -2.00143
c 0.73060 0.06328 -0.74392
c 2.75192 0.65035 -2.07459
c 3.53269 0.64859 -0.94444
c 2.99939 0.17781 0.30243
c 1.60615 -0.18058 0.39953
c 3.85458 -0.02233 1.42329
c 3.39008 -0.63385 2.58043
c 2.05200 -1.09356 2.63696
c 1.18512 -0.87034 1.57282
h 3.17896 0.90592 -3.05718
h 4.59536 0.93286 -0.99140
h -4.55093 -1.00024 -0.99579
h -3.11207 -0.93155 -3.07051
h -4.88635 -0.34122 1.33445
h -4.07007 0.76741 3.42495
h -1.71725 1.66706 3.50976
h -0.17505 1.31355 1.61590
h 4.06646 -0.79358 3.43384

h 4.90833 0.28659 1.33972
h 1.06884 0.40011 -4.16843
h 1.69406 -1.63901 3.52322
h 0.15859 -1.25375 1.62948
Molecule 8(H+)
35
c -3.42954 0.62125 2.54095
c -2.10404 1.11067 2.62702
c -1.21001 0.90601 1.58214
c -1.60568 0.21695 0.39973
c -2.98806 -0.18095 0.27454
c -3.86507 -0.00362 1.37551
c -3.49079 -0.67587 -0.98132
c -2.71148 -0.65756 -2.10722
c -1.33206 -0.26558 -2.01154
c -0.71379 -0.00323 -0.72371
n -0.67022 -0.13888 -3.17995
n 0.58006 0.22406 -3.10547
c 1.35204 0.30042 -1.97504
c 0.72495 0.03558 -0.71146
c 2.73205 0.63719 -2.09730
c 3.51948 0.62311 -0.97152
c 3.00832 0.15777 0.28792
c 1.61668 -0.19785 0.41867
c 3.88886 -0.03621 1.38541
c 3.44232 -0.63740 2.55787
c 2.10407 -1.08571 2.64963
c 1.20950 -0.86522 1.60593
h 3.14474 0.90052 -3.08300
h 4.58112 0.90482 -1.03928
h -4.54308 -0.99273 -1.03839
h -3.09909 -0.91504 -3.10263
h -4.90952 -0.33648 1.27986
h -4.12415 0.76488 3.38197
h -1.77960 1.66340 3.52087
h -0.19253 1.31032 1.66025
h 4.13537 -0.79467 3.39747
h 4.94203 0.26474 1.27946
h 1.02448 0.40337 -4.01701
h 1.76514 -1.62023 3.54930
h 0.18164 -1.23865 1.69603
Molecule 8
34
c -3.45516 0.62389 2.55776
c -2.11896 1.08045 2.65747
c -1.22473 0.87134 1.61262
c -1.61911 0.19971 0.42081
c -3.00664 -0.16482 0.28258
c -3.89009 0.02442 1.38196
c -3.50256 -0.63713 -0.98170
c -2.69630 -0.63315 -2.09220
c -1.31095 -0.28050 -1.98289
c -0.71937 -0.02610 -0.70462
n -0.61753 -0.19194 -3.16965
n 0.61787 0.19159 -3.16960
c 1.31117 0.28023 -1.98277
c 0.71945 0.02593 -0.70454
c 2.69652 0.63287 -2.09196
c 3.50267 0.63693 -0.98138
c 3.00662 0.16472 0.28289
c 1.61907 -0.19980 0.42099

c 3.88993 -0.02438 1.38240
c 3.45488 -0.62376 2.55820
c 2.11867 -1.08032 2.65780
c 1.22455 -0.87128 1.61284
h 3.06563 0.88360 -3.09706
h 4.56142 0.93008 -1.05529
h -4.56129 -0.93029 -1.05570
h -3.06529 -0.88398 -3.09732
h -4.94104 -0.28459 1.26922
h -4.15393 0.77401 3.39470
h -1.78405 1.61130 3.56136
h -0.19890 1.25214 1.70007
h 4.15357 -0.77383 3.39522
h 4.94088 0.28471 1.26978
h 1.78367 -1.61112 3.56168
h 0.19869 -1.25202 1.70024
Molecule 4(H+)
42
c 3.13640 -2.42896 -0.52141
c 2.83864 -1.11895 -0.05041
c 1.58985 -0.49831 -0.41697
c 0.76250 -1.18345 -1.35405
c 1.09500 -2.44848 -1.82767
c 2.27327 -3.09532 -1.38267
c 1.26419 0.81995 0.12630
c 2.33779 1.58206 0.69959
c 3.57980 0.94127 1.02373
c 3.79777 -0.38117 0.72040
c -0.04310 1.45165 0.03479
c -0.09117 2.88933 0.05042
c 1.04755 3.63757 0.48232
c 2.18974 2.99059 0.89669
c -1.32662 0.76422 -0.02088
c -2.48809 1.46902 -0.49124
c -2.41482 2.87466 -0.72082
c -1.27824 3.56870 -0.36550
c -1.58220 -0.54516 0.52577
c -2.85241 -1.20528 0.38389
c -3.91688 -0.51960 -0.28210
c -3.73982 0.78676 -0.66758
c -3.01499 -2.49757 0.94093
c -1.98698 -3.11518 1.65719
c -0.78583 -2.42107 1.82195
n -0.63439 -1.20368 1.27034
h -0.13987 -0.69007 -1.74004
h 2.52405 -4.10160 -1.74989
h 4.08796 -2.89241 -0.21812
h 0.97694 4.73501 0.51633
h 3.03536 3.55723 1.31497
h -4.87903 -1.03282 -0.42315
h -4.57629 1.35076 -1.10755
h -3.98138 -3.00934 0.81222
h -2.10613 -4.11416 2.09701
h 0.07441 -2.81425 2.38165
h 4.74222 -0.87183 1.00131
h 4.35967 1.53721 1.52146
h -3.30325 3.40148 -1.09876
h -1.25963 4.66811 -0.41635
h 0.44629 -2.93957 -2.56856
h 0.27874 -0.73063 1.36276
Molecule 4

41
c -1.91588 -3.11754 1.58726
c -0.75911 -2.33369 1.83021
n -0.58079 -1.11485 1.34135
c -1.54814 -0.55696 0.56944
c -2.80311 -1.22647 0.34638
c -2.94676 -2.54532 0.85515
c -3.86807 -0.54622 -0.32760
c -3.71992 0.77300 -0.68262
c -2.48071 1.47086 -0.48007
c -1.33105 0.77371 0.01130
c -2.41053 2.88101 -0.71180
c -1.27740 3.58152 -0.36566
c -0.08890 2.90096 0.04858
c -0.05290 1.46337 0.05675
c 1.05962 3.64451 0.46440
c 2.20139 2.99160 0.87041
c 2.33374 1.57904 0.68572
c 1.24899 0.82360 0.13830
c 3.57198 0.92587 1.00677
c 3.77394 -0.39925 0.70959
c 2.79979 -1.12746 -0.05202
c 1.55359 -0.49724 -0.39760
c 0.71803 -1.16840 -1.33438
c 1.03020 -2.43534 -1.81469
c 2.20491 -3.09631 -1.38259
c 3.08063 -2.43902 -0.52748
h -0.18850 -0.66747 -1.69870
h 2.44021 -4.10680 -1.75033
h 4.02862 -2.91339 -0.22727
h 0.99528 4.74328 0.49181
h 3.05860 3.55327 1.27351
h -4.81653 -1.07808 -0.50284
h -4.56092 1.32932 -1.12565
h -3.88783 -3.08961 0.67496
h -1.99559 -4.13604 1.99546
h 0.05590 -2.73245 2.46220
h 4.71462 -0.90135 0.98525
h 4.35941 1.51728 1.49989
h -3.30347 3.40160 -1.09087
h -1.25723 4.68106 -0.42363
h 0.36126 -2.92119 -2.54148
Molecule 11(H+)
42
c 3.09945 -2.43050 -0.51634
c 2.82539 -1.10510 -0.07376
c 1.57111 -0.48428 -0.41620
c 0.71150 -1.19104 -1.30662
c 1.01764 -2.47307 -1.75347
c 2.20463 -3.11678 -1.32804
c 1.27237 0.84805 0.10234
c 2.36311 1.61187 0.63106
c 3.61331 0.97236 0.92902
c 3.81438 -0.35707 0.64845
c -0.03631 1.48359 0.04894
c -0.08405 2.92390 0.05659
c 1.06951 3.67212 0.44634
c 2.22437 3.02389 0.82049
c -1.31590 0.79913 0.02533
c -2.47755 1.49974 -0.44428
c -2.42300 2.91011 -0.65809

c -1.28297 3.60560 -0.32109
c -1.55137 -0.53309 0.55913
c -2.79626 -1.22640 0.30672
c -3.84833 -0.54888 -0.37233
c -3.70074 0.78858 -0.67521
c -2.95074 -2.56058 0.78437
c -1.97000 -3.15364 1.54805
n -0.85600 -2.42132 1.86594
c -0.63113 -1.17621 1.40748
h -0.19491 -0.69921 -1.68380
h 2.43814 -4.13418 -1.67585
h 4.05822 -2.89105 -0.23142
h 1.00237 4.76979 0.47852
h 3.08639 3.59136 1.20305
h -4.78690 -1.08006 -0.58673
h -4.54349 1.34573 -1.11365
h -3.87081 -3.11917 0.56104
h -2.02797 -4.17342 1.94998
h 4.76459 -0.84768 0.90947
h 4.41055 1.57460 1.39041
h -3.31942 3.43231 -1.02371
h -1.26636 4.70518 -0.36621
h 0.34334 -2.97772 -2.46192
h 0.28626 -0.68718 1.75578
h -0.15077 -2.84174 2.48044
Molecule 11
41
c 3.08706 -2.42218 -0.56911
c 2.81468 -1.10806 -0.09603
c 1.55706 -0.48299 -0.41300
c 0.69149 -1.17098 -1.31030
c 0.99542 -2.44111 -1.78740
c 2.18752 -3.09108 -1.38865
c 1.26196 0.84078 0.12620
c 2.35695 1.59739 0.65466
c 3.60521 0.94914 0.94210
c 3.80528 -0.37498 0.63784
c -0.04651 1.47595 0.07622
c -0.08549 2.91431 0.07330
c 1.06811 3.65732 0.47649
c 2.22104 3.00764 0.85424
c -1.32391 0.78102 0.04005
c -2.46374 1.47674 -0.47808
c -2.40187 2.88917 -0.69282
c -1.27703 3.59502 -0.32979
c -1.55458 -0.54780 0.58865
c -2.78162 -1.23651 0.30659
c -3.82060 -0.56738 -0.42089
c -3.68160 0.76114 -0.74174
c -2.94145 -2.55251 0.81793
c -1.94918 -3.09648 1.62321
n -0.84669 -2.40747 2.00697
c -0.66660 -1.19318 1.50349
h -0.22746 -0.67912 -1.65296
h 2.41398 -4.10523 -1.75126
h 4.04437 -2.89085 -0.29139
h 0.99992 4.75552 0.51421
h 3.08325 3.57044 1.24447
h -4.74803 -1.11344 -0.65244
h -4.51194 1.31083 -1.21274
h -3.86097 -3.11664 0.59619

h -2.04628 -4.12301 2.01911
h 4.75387 -0.87375 0.89063
h 4.40212 1.54270 1.41674
h -3.29330 3.40604 -1.08066
h -1.26242 4.69499 -0.37603
h 0.30443 -2.93812 -2.48515
h 0.22077 -0.64650 1.86291
Molecule 6(H+)
47
n 1.19337 -0.28198 1.68159
c 1.66281 0.02639 0.44156
c 3.08830 0.13200 0.29027
c 3.90238 -0.22830 1.39310
c 3.35746 -0.61660 2.61397
c 1.96319 -0.58907 2.72907
c 0.73465 0.14655 -0.70085
c 1.38376 0.38958 -1.95859
c 2.79328 0.78431 -2.06481
c 3.66409 0.62130 -0.95101
c -0.72889 -0.08564 -0.70071
c -1.37396 -0.35717 -1.95473
c -0.64657 -0.20716 -3.16379
c 0.66060 0.21148 -3.16635
c -1.66067 0.06026 0.43566
c -3.08566 -0.04951 0.28260
c -3.65759 -0.56598 -0.94942
c -2.78334 -0.75366 -2.05664
c -3.90313 0.33446 1.37485
c -3.36206 0.75044 2.58830
c -1.96811 0.72716 2.70786
n -1.19512 0.39736 1.66964
c 3.33056 1.32518 -3.26377
c 5.03531 0.96291 -1.07495
c -5.02859 -0.90963 -1.07026
c -3.31711 -1.32054 -3.24511
h -4.99511 0.30877 1.26090
h -3.99717 1.05668 3.43057
h -1.44953 0.96866 3.64974
h 3.98982 -0.90477 3.46464
h 4.99469 -0.20621 1.28155
h -1.14699 -0.37196 -4.12508
h 1.16451 0.35402 -4.12940
h 1.44185 -0.80872 3.67476
c -4.66317 -1.66402 -3.34271
c -5.53017 -1.44737 -2.25170
h -2.66444 -1.53020 -4.10255
h -5.04484 -2.11536 -4.27042
h -6.59328 -1.71959 -2.32491
h -5.70941 -0.78144 -0.21812
c 5.54038 1.47494 -2.26628
c 4.67679 1.66708 -3.36457
h 6.60357 1.74614 -2.34205
h 5.06129 2.09806 -4.30077
h 2.68047 1.51563 -4.12764
h 5.71324 0.85397 -0.21785
h -0.00127 0.05966 1.80590
Molecule 6
46
n 0.96002 -0.93617 1.31667
c 1.53569 -0.22453 0.31668
c 2.92242 0.12437 0.33629

c 3.64282 -0.17516 1.52052
c 3.01559 -0.83352 2.57239
c 1.66971 -1.22867 2.39792
c 0.72638 0.05849 -0.87337
c 1.40516 0.32098 -2.09513
c 2.81747 0.71669 -2.08152
c 3.55293 0.69405 -0.85348
c -0.71086 -0.05098 -0.87269
c -1.39072 -0.31449 -2.09363
c -0.65713 -0.19012 -3.30835
c 0.67050 0.19558 -3.30910
c -1.51912 0.23327 0.31777
c -2.90590 -0.11537 0.33887
c -3.53752 -0.68599 -0.84985
c -2.80309 -0.70989 -2.07847
c -3.62513 0.18508 1.52356
c -2.99679 0.84399 2.57445
c -1.65101 1.23881 2.39846
n -0.94240 0.94551 1.31672
c 3.47223 1.18440 -3.25117
c 4.88831 1.17178 -0.84470
c -4.87298 -1.16351 -0.83953
c -3.45888 -1.17852 -3.24719
h -4.69090 -0.07275 1.60374
h -3.53942 1.09100 3.49920
h -1.13977 1.83479 3.17727
h 3.55913 -1.07992 3.49678
h 4.70859 0.08305 1.59943
h -1.15966 -0.34863 -4.27185
h 1.17228 0.35326 -4.27313
h 1.15931 -1.82423 3.17760
c -4.77603 -1.63183 -3.21484
c -5.48840 -1.63179 -1.99841
h -2.91392 -1.21565 -4.20047
h -5.25120 -2.00094 -4.13638
h -6.52398 -2.00222 -1.96103
h -5.43855 -1.17290 0.10273
c 5.50271 1.63915 -2.00450
c 4.78934 1.63799 -3.22033
h 6.53826 2.00977 -1.96815
h 5.26350 2.00635 -4.14268
h 2.92657 1.22059 -4.20409
h 5.45470 1.18207 0.09706
Molecule 9(H+)
73
c 0.48375 2.99473 2.59357
c -0.27070 2.70053 1.43241
n -1.20483 3.61214 1.03475
c -1.40310 4.81573 1.62952
c -0.65478 5.15219 2.74793
c 0.28450 4.22057 3.23767
c -0.13346 1.43327 0.69400
c -0.11395 0.21511 1.42574
c -0.03637 -1.00882 0.71442
c -0.00691 -1.00810 -0.71190
c 0.06382 0.21779 -1.43068
c 0.00159 1.44303 -0.72278
c -0.02386 -2.30857 -1.44462
c 1.01856 -3.24732 -1.30682
c 0.96729 -4.47322 -1.98299
c -0.12687 -4.81334 -2.80444

c -1.16687 -3.86714 -2.93954
c -1.11700 -2.63433 -2.27975
c -0.19444 -6.13767 -3.52100
c -0.00682 -2.31037 1.44911
c 1.11634 -2.66540 2.22751
c 1.16899 -3.90400 2.87994
c 0.10417 -4.82546 2.78915
c -1.01780 -4.45836 2.01620
c -1.07464 -3.22620 1.35346
c -0.30837 0.19999 2.94202
o 0.75268 0.84401 3.68441
c 1.43298 1.92730 3.08551
c 0.17966 2.74021 -1.43955
c -0.46674 2.96522 -2.68143
c -0.21905 4.18667 -3.33052
c 0.63150 5.12713 -2.73360
c 1.20171 4.81351 -1.48704
n 0.98103 3.65462 -0.85097
c 0.38952 0.18887 -2.92759
c 1.79925 0.71674 -3.24115
c 0.15259 -6.14550 3.51528
c -1.34130 1.88013 -3.26633
o -0.61326 0.78086 -3.77418
c -1.69187 0.68868 3.39659
h 1.98261 1.75221 -2.89865
h 1.95016 0.68378 -4.33759
h -0.69220 4.39470 -4.30340
h 0.85154 6.08828 -3.22105
h -0.23431 -0.85470 3.26349
h 2.06800 1.59941 2.22680
h 2.10445 2.33568 3.86605
h -2.47464 0.01736 2.98998
h -1.93436 1.72356 3.08718
h -1.73913 0.64581 4.50203
h 0.87651 4.46016 4.13484
h -0.81263 6.12235 3.23809
h 1.87070 5.53129 -0.98072
h -2.17049 5.46505 1.18614
h 0.38263 -0.87203 -3.23669
h -2.07182 1.54480 -2.48616
h -1.92884 2.27310 -4.12007
h 2.55503 0.05892 -2.76695
h 1.96116 -1.96398 2.31377
h 2.06077 -4.16493 3.47118
h -1.86317 -5.15819 1.92407
h -1.95300 -2.97437 0.74072
h -1.93556 -1.90901 -2.40905
h -2.03348 -4.10316 -3.57711
h 1.79701 -5.18754 -1.86521
h 1.87855 -3.01494 -0.66194
h 0.69856 -6.76023 -3.32265
h -1.08930 -6.71605 -3.20950
h -0.27425 -5.99558 -4.61884
h -0.45162 -6.91853 3.00137
h 1.18966 -6.52216 3.61298
h -0.25487 -6.04405 4.54435
h -1.74982 3.37959 0.19518
Molecule 9
72
c 1.07158 -3.22810 -1.34099
c 0.00844 -2.30955 -1.44272

c -1.10545 -2.66610 -2.23551
c -1.15365 -3.90200 -2.89136
c -0.09374 -4.82825 -2.78552
c 1.01932 -4.46302 -2.00307
c 0.02719 -1.00452 -0.70970
c -0.02401 -1.00562 0.71156
c -0.10823 0.21948 1.42423
c -0.08446 1.44058 0.70651
c 0.08525 1.44171 -0.70090
c 0.11007 0.22178 -1.42050
c -0.33880 0.19957 2.93662
c -1.72913 0.71275 3.35049
c -0.00418 -2.31171 1.44277
c 1.11062 -2.67110 2.23089
c 1.16021 -3.91077 2.88203
c 0.09949 -4.83446 2.77801
c -1.01467 -4.46741 1.99565
c -1.06780 -3.23116 1.33865
c 0.33876 0.20449 -2.93321
o -0.71802 0.81531 -3.70087
c -1.39071 1.92999 -3.12947
c -0.45866 2.99396 -2.60646
c 0.27105 2.73682 -1.41922
n 1.17282 3.59855 -0.90927
c 1.36534 4.76102 -1.54598
c 0.68259 5.12627 -2.71888
c -0.23866 4.21658 -3.25701
c -0.27316 2.73436 1.42647
n -1.17536 3.59557 0.91635
c -1.37025 4.75697 1.55430
c -0.69018 5.12126 2.72904
c 0.23160 4.21213 3.26724
c 0.45438 2.99082 2.61518
c 1.38753 1.92762 3.13782
o 0.71578 0.81113 3.70671
c 0.14301 -6.15659 3.50400
c -0.16120 -6.16035 -3.49130
c 1.72764 0.72055 -3.34828
h 1.94502 1.74783 -3.00236
h 1.79632 0.70388 -4.45399
h -0.79031 4.45207 -4.18171
h 0.87712 6.09648 -3.20023
h -0.29809 -0.85777 3.25831
h 2.06175 1.60392 2.30707
h 2.02565 2.33245 3.94938
h -2.50850 0.03913 2.93930
h -1.94786 1.73993 3.00513
h -1.79912 0.69507 4.45610
h 0.78126 4.44692 4.19329
h -0.88739 6.09017 3.21192
h 2.10974 5.44508 -1.09944
h -2.11448 5.44090 1.10729
h 0.29956 -0.85242 -3.25652
h -2.06325 1.60396 -2.29826
h -2.03061 2.33500 -3.93953
h 2.50868 0.04793 -2.93863
h 1.94753 -1.96219 2.33112
h 2.04610 -4.16958 3.48426
h -1.85773 -5.16957 1.89289
h -1.94214 -2.97595 0.72145
h -1.94016 -1.95480 -2.33751

h -2.03585 -4.15551 -3.50144
h 1.86419 -5.16362 -1.90616
h 1.94689 -2.97403 -0.72476
h 0.75037 -6.76428 -3.31658
h -1.03265 -6.75588 -3.14627
h -0.27738 -6.03148 -4.58804
h -0.45497 -6.93026 2.98258
h 1.18019 -6.53333 3.60733
h -0.27160 -6.06170 4.53133
Molecule 10(H+)
74
c -3.67163 0.05748 0.96517
c -3.66371 1.47005 1.01210
c -4.89639 2.17338 1.06647
c -6.10275 1.44960 1.04034
c -6.10113 0.04841 0.96765
c -4.88110 -0.64929 0.94266
c -2.38579 2.24140 1.04094
c -1.36118 1.99843 0.07713
c -0.09602 2.65118 0.17307
c 0.12373 3.56941 1.22781
c -0.93734 3.89981 2.12445
c -2.18308 3.21791 2.04640
c -1.60094 1.09332 -1.05780
c -0.64071 0.15958 -1.52264
c -0.95685 -0.65287 -2.61973
c -2.21141 -0.56099 -3.25517
c -3.13079 0.36058 -2.77231
n -2.79580 1.14620 -1.72046
c 0.69412 0.11204 -0.81346
o 1.50747 1.21911 -1.12298
c 0.95026 2.53878 -0.93865
c 0.48309 3.13973 -2.27319
c 1.45376 4.23450 1.38053
c 2.58354 3.47935 1.76663
c 3.82726 4.09635 1.94345
c 3.99273 5.48319 1.73540
c 2.86219 6.22685 1.33996
c 1.61147 5.61855 1.16949
c -0.72112 4.96960 3.14156
c 0.25055 4.83598 4.15380
c 0.45707 5.86369 5.08291
c -0.28003 7.06414 5.02601
c -1.25065 7.19210 4.00788
c -1.47608 6.16428 3.08620
c -3.26393 3.48266 3.09956
o -4.45973 4.07103 2.54860
c -4.86084 3.67528 1.24747
c -0.05160 8.17889 6.01401
c 5.33708 6.13514 1.93327
c -3.56567 2.28752 4.01727
h -3.91547 1.38221 3.48928
h -4.35211 2.59178 4.73483
h -7.05733 1.99674 1.09113
h -7.05322 -0.50305 0.95213
h 1.82172 3.13756 -0.61671
h 0.50706 0.02993 0.28602
h 1.26257 -0.78603 -1.12593
h 0.27522 4.22072 -2.14320
h -0.42572 2.66410 -2.68999
h 1.29560 3.02987 -3.01737

h -0.21366 -1.38189 -2.97881
h -2.47292 -1.19842 -4.11053
h -4.87351 -1.74940 0.92135
h -4.13733 0.50121 -3.19010
h -2.87796 4.27944 3.76103
h -4.18796 4.12822 0.47495
h -5.86875 4.11148 1.10117
h -2.65593 2.02587 4.59503
h 2.47966 2.39535 1.93272
h 4.69203 3.48793 2.25246
h 2.96268 7.30984 1.16702
h 0.74322 6.22768 0.87672
h -2.24857 6.28328 2.31006
h -1.84424 8.11744 3.93939
h 1.21487 5.73179 5.87106
h 0.85065 3.91631 4.21475
h 0.72613 7.91918 6.75683
h 0.26564 9.11016 5.49979
h -0.98337 8.42244 6.56595
h 5.30458 7.22077 1.72119
h 5.69621 6.00288 2.97522
h 6.10464 5.68156 1.27210
h -2.71521 -0.48849 0.97263
h -3.50547 1.78694 -1.33669
Molecule 10
73
c -0.69929 0.15083 -1.50033
c -1.65477 1.12670 -1.11646
n -2.81630 1.31299 -1.77487
c -3.08319 0.52605 -2.82428
c -2.21666 -0.48398 -3.27793
c -1.00078 -0.66207 -2.60403
c -1.37831 2.02117 0.04739
c -0.12003 2.66850 0.14818
c 0.10322 3.59291 1.20051
c -0.94881 3.91211 2.10271
c -2.18670 3.22172 2.03275
c -2.38752 2.24544 1.02213
c -0.72649 4.97897 3.12959
c 0.19869 4.81155 4.17800
c 0.42280 5.83647 5.10822
c -0.26133 7.06491 5.02348
c -1.19189 7.22650 3.97466
c -1.42356 6.20512 3.04596
c -0.01814 8.17599 6.01541
c 1.43455 4.25801 1.36109
c 2.56287 3.50712 1.75925
c 3.80459 4.12400 1.95203
c 3.97081 5.51129 1.75235
c 2.84496 6.25361 1.34514
c 1.59809 5.64167 1.15427
c 0.95057 2.48874 -0.93075
o 1.48483 1.15134 -1.02758
c 0.60684 0.07181 -0.74803
c -3.65165 1.45427 1.00372
c -4.89603 2.12157 1.11503
c -6.08531 1.37354 1.13413
c -6.05707 -0.02661 1.05152
c -4.82260 -0.69132 0.96501
c -3.63111 0.04508 0.94240
c -3.25774 3.48910 3.09363

c -3.53197 2.29639 4.02687
c 5.31293 6.16554 1.96994
c -4.88967 3.62264 1.27109
o -4.46938 4.05811 2.56140
c 0.53489 3.04242 -2.30445
h -3.84538 1.37732 3.49953
h -4.33631 2.58201 4.73305
h -7.04860 1.90184 1.21960
h -6.99639 -0.60041 1.06638
h 1.82983 3.08203 -0.61732
h 0.39260 0.01181 0.34819
h 1.16456 -0.84471 -1.02938
h 0.40205 4.14125 -2.23365
h -0.40358 2.60938 -2.69643
h 1.34511 2.83399 -3.03077
h -0.28116 -1.43044 -2.93004
h -2.48742 -1.10668 -4.14380
h -4.78726 -1.79056 0.91841
h -4.04423 0.70739 -3.33885
h -2.87272 4.29534 3.74561
h -4.24201 4.06753 0.47624
h -5.91038 4.03554 1.14148
h -2.61814 2.06895 4.61370
h 2.45655 2.42174 1.91313
h 4.66737 3.51453 2.26639
h 2.94556 7.33802 1.17767
h 0.73226 6.24768 0.84856
h -2.16319 6.34776 2.24247
h -1.74736 8.17413 3.88513
h 1.15179 5.67887 5.91935
h 0.75635 3.86671 4.25923
h 0.72746 7.88684 6.78104
h 0.35370 9.09271 5.51095
h -0.95350 8.45948 6.54249
h 5.27816 7.25284 1.76331
h 5.66209 6.03138 3.01551
h 6.09103 5.72089 1.31428
h -2.66386 -0.47680 0.88405
Molecule 12(H+)
51
n -0.94848 0.72895 1.64786
c -1.44327 0.09453 0.55207
c -2.80142 -0.30655 0.59858
c -3.52919 -0.11778 1.77943
c -2.94247 0.49575 2.90080
c -1.63040 0.93691 2.79807
c -3.39044 -0.88942 -0.64987
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h -5.15726 3.02065 -2.63231
h -4.03369 4.28592 -1.96865
h -3.67845 3.44847 -3.53038

Chapter 3

Manuscript-II

Inhibition of 5-lipoxygenase as Anti-inflammatory Mode of Action of *Plectranthus zeylanicus* Benth & Chemical Characterization of Ingredients by a Mass Spectrometric Approach

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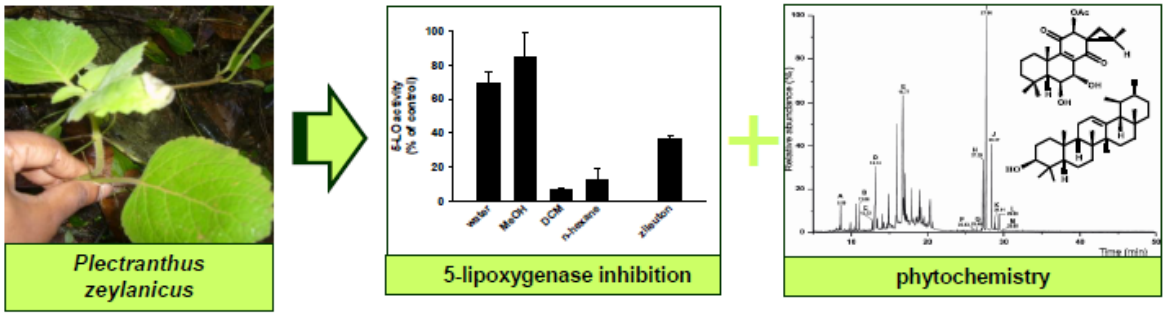

Abstract

Ethnopharmacological relevance: The perennial herb *Plectranthus zeylanicus* Benth is extensively used in traditional medicine in Sri Lanka and South India for treating inflammatory conditions, but pharmacological features of *P. zeylanicus* are hardly explored in order to understand and rationalize its use in ethnomedicine. As 5-lipoxygenase (5-LO) is a key enzyme in inflammatory disorders, we investigated 5-LO inhibition by *P. zeylanicus* extracts and analyzed relevant constituents.

Materials and Methods: We applied cell-free and cell-based assays to investigate suppression of 5-LO activity. Cell viability, radical scavenger activities, and inhibition of reactive oxygen species formation (ROS) in neutrophils were analysed to exclude unspecific cytotoxic or antioxidant effects. Constituents of the extracts were characterized by bioassay-guided fractionation and by analysis using gas or liquid chromatography coupled to mass spectrometric (Orbitrap) analysis.

Results: Extracts (*n*-hexane or dichloromethane) of *P. zeylanicus* potently suppressed 5-LO activity in stimulated human neutrophils (IC_{50} = 6.6-12 μg/ml) and inhibited isolated human recombinant 5-LO (IC_{50} = 0.7-1.2 μg/ml). In contrast, no significant radical scavenging activity or suppression of ROS formation was observed, and neutrophil viability was unaffected. Besides ubiquitously occurring ingredients, coleone P, cinncassiol A and C, and callistric acid were identified as constituents in the most active fraction.

Conclusions: Together, potent inhibition of 5-LO activity, without concomitant anti-oxidant activity and cytotoxic effects, rationalizes the ethnopharmacological use of *P. zeylanicus* as anti-inflammatory remedy. Modern chromatographic/mass spectrometric analysis reveals discrete chemical structures of relevant constituents.

Keywords: *Plectranthus zeylanicus*, inflammation, 5-lipoxygenase, neutrophils, radical scavenger, mass spectrometry.

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Inhibition of 5-lipoxygenase as anti-inflammatory mode of action of *Plectranthus zeylanicus* Benth and chemical characterization of ingredients by a mass spectrometric approach

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ABSTRACT

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Materials and methods: We applied cell-free and cell-based assays to investigate suppression of 5-LO activity. Cell viability, radical scavenger activities, and inhibition of reactive oxygen species formation (ROS) in neutrophils were analysed to exclude unspecific cytotoxic or antioxidant effects. Constituents of the extracts were characterized by bioassay-guided fractionation and by analysis using gas or liquid chromatography coupled to mass spectrometric (Orbitrap) analysis.

Results: Extracts of *Plectranthus zeylanicus* prepared with *n*-hexane or dichloromethane potently suppressed 5-LO activity in stimulated human neutrophils (IC_{50}=6.6 and 12 μg/ml, respectively) and inhibited isolated human recombinant 5-LO (IC_{50}=0.7 and 1.2 μg/ml, respectively). In contrast, no significant radical scavenging activity or suppression of ROS formation was observed, and neutrophil viability was unaffected. Besides ubiquitously occurring ingredients, coleone P, cinncassiol A and C, and callistric acid were identified as constituents in the most active fraction.

Conclusions: Together, potent inhibition of 5-LO activity, without concomitant anti-oxidant activity and cytotoxic effects, rationalizes the ethnopharmacological use of *Plectranthus zeylanicus* as anti-inflammatory remedy. Modern chromatographic/mass spectrometric analysis reveals discrete chemical structures of relevant constituents.

1. Introduction

Plants and their products have been systematically used in Sri Lanka for treating illnesses for over thousand years. Even though modern health care facilities are readily available in most part of the country, many people still rely on indigenous medicines for certain illnesses such as common cold, body aches, minor fractures, etc. Among the native flora of Sri Lanka, more than 1400 plants are used in indigenous medicine (Wijesundera, 2004).

Plectranthus zeylanicus Benth (synonym *Coleus zeylanicus* (Benth) Cramer), locally known as Iruveriya, is a perennial herb of the family Lamiaceae, which is extensively used in traditional medicine (Dassanayake and Fosberg, 1981). Although it is claimed to be an endemic species to Sri Lanka, it can be easily grown in prevailing climatic and soil conditions in the country. The plant is also introduced to South India where it is widely cultivated as a pot herb in home gardens (Sivarajan and Balachandran, 1986). The plant has aromatic, astringent and stomachic properties and is used in folk medicine in decoctions for fevers, dysentery, diarrhea, vomiting and thirst. It acts as a cholagogue but has been also used also as a diuretic and diaphoretic and as an antidote for tarantula bites (Jayaweera, 1982). According to the pharmacopoeia, *Plectranthus zeylanicus* is used as a constituent of various ayurvedic and traditional medicinal preparations (as powders, called Kalkaya, and as oils, called Prameha

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or Kvathaya) and the plant is described to be effective in combating various conditions like asthma, common cold, varieties of fever, cough, leucoderma, diarrhea, chronic liver diseases, chronic ulcers, burning sensation of head, dental diseases, eye diseases and others (Ayurveda Pharmacopeia, 1979).

Despite the wide array of usages in traditional and folk medicine, the bioactivities of *Plectranthus zeylanicus* are hardly explored in order to understand and rationalize the reported ethnopharmacological use of the plant. The in vitro microbicidal activity of essential oils of *Plectranthus zeylanicus* was studied using several bacteria and fungi revealing an inhibitory activity against *Proteus vulgaris, Aspergillus parasiticus, Aspergillus niger, Rhizopus oryzae and Colletotrichum musae* (Deena et al., 2002). The aqueous extract of the plant demonstrated a high inhibitory activity of the terminal route of the complement cascade, thus suggesting the possible use of the extract and/or its active principle(s) in the therapy of septic shock (Beukelman et al., 1994). Extracts of the stem and leaves exhibit antioxidant activities (Rasineni et al., 2008) while the essential oil of the plant showed insecticidal activity against the stored grain pest *Callosobruchus maculatus*, suggesting the plant as an alternative pest control agent with low toxicity to warm blooded mammals (Balachandra et al., 2012).

Although diterpenoids, essential oils, phenolics, and few triterpenoids were isolated from different species of the genus *Plectranthus*, the phytochemistry of this genus is far from being established. In the case of *Plectranthus zeylanicus*, the ethanolic extract afforded abietane-type diterpenoides characterized as 7β-acetoxy-6β-hyroxyroyleanone, 7β-, 6β-dihyroxyroyleanone, and 7α-acetoxy-6β-hydroxyroyleanone (Mehrotra et al., 1989). Geraniol, geranyl acetate, caryophyllene, eudesm-7(11)-en-4-ol, p-cymene, fenchyl acetate, fenchyl formate, and bornyl acetate were identified in the essential oils of aerial parts and roots of *Plectranthus zeylanicus* grown in Sri Lanka (Arambewela and Wijesinghe, 2006), while α-terpeneol was identified as the major component of the essential oil of an Indian variety (Arambewela and Wijesinghe, 2006). Furthermore, peaks corresponding to caffeic acid and coumaric acid were identified by the RP-HPLC-UV spectral analysis of a water/methanol extract of the leaves (Rasineni et al., 2008). However, the current knowledge about the phytochemistry and in particular about the bioactive metabolites in *Plectranthus zeylanicus* is insufficient to rationalize its use in traditional medicine, thus the present study was undertaken to address this aspect.

The leukotrienes (LTs) are crucial mediators of inflammatory and allergic reactions involved in the pathophysiology of for example asthma, allergic rhinitis, atherosclerosis, and cancer (Werz and Steinhilber, 2006). 5-Lipoxygenase (5-LO) catalyzes the first two key steps in LT biosynthesis from arachidonic acid and is considered as attractive drug target (Radmark et al., 2007; Pergola and Werz, 2010). In fact, several natural products from plants used as anti-inflammatory remedies were shown to suppress the formation of LTs, most of them by inhibiting 5-LO activity (Werz, 2007). Mechanistically, many natural products of plant origin (flavonoids, polyphenols, coumarins) interfere with 5-LO activity due anti-oxidant activities, that is, by uncoupling of the redox cycle of the 5-LO active-site iron (Werz, 2007).

Isolation and identification of secondary metabolites, which involves tedious and time consuming purification steps, is the main bottle-neck in natural products chemistry. Therefore, development of new methodologies that facilitate rapid identification of secondary metabolites from natural product mixtures has become a crucial requirement. The advances made in separation technologies and mass spectrometric methods over the past few years have largely revolutionized and tremendously accelerated the compound identification process. Mass spectrometry (MS), coupled to HPLC or UPLC combined with MS/MS data bases have become indispensable tools in structural characterization of small molecules. The present investigation was carried out in order to evaluate 5-LO inhibition as anti-inflammatory mode of action of *Plectranthus zeylanicus* and novel MS methodologies were applied as means to identify related constituents.

2. Materials and methods

2.1. Plant material

Plectranthus zeylanicus plants were collected in Nittambuwa (Gampaha district—Western Province of Sri Lanka) in 2011/2012. The plant was identified by the author (MN), a botanist, and confirmed based on the books "A Revised Handbook to the Flora of Ceylon: volume—III, M.D. Dassanayake & F.R. Fosberg" and "Medicinal plants (indigenous and exotic) used in Ceylon: Volume 2 by D.M.A. Jayaweera", and authenticated by comparison with the herbarium specimens at the National herbarium, Royal Botanical Garden, Peradeniya, Sri Lanka. A voucher specimen (Plec-WP-1-1206) is deposited at the Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Sri Lanka.

2.2. Preparation of crude extracts

The plant materials (whole plant) were thoroughly washed and dried in shade (30 ± 2 °C) for six days. Dried plants were powdered using an electrical grinder (Singer, model KA-MIXEE). Thirteen gram of powdered material was successively extracted with 600 ml of *n*-hexane, dichloromethane (DCM), ethyl acetate, or methanol (Roth, Karlsruhe, Germany) at room temperature using a linear shaker for 20 min. In addition, 3.0 g of powdered material was extracted in 300 ml of 70% methanol/water in the presence of 0.05% acetic acid by heating for 2 h at 60 °C. Though this solvent mixture may cause unstable or degraded products due to hydrolysis, it might extract most of the phenolic compounds potentially responsible for bioactivity. The extracts were evaporated into dryness with the use of rotary evaporator (BÜCHI, R-114, Germany). For bioactivity studies, extracts were freshly solubilized with DMSO (30 mg/ml), except the aqueous extract that was solubilized in water, further diluted by solvent, and immediately used for experiments.

2.3. Evaluation of bioactivity

2.3.1. 5-Lipoxygenase (5-LO) activity in intact neutrophils and whole Blood

Human neutrophils were freshly isolated from leukocyte concentrates obtained at the University Hospital Jena, Germany, as described (Pergola et al., 2008). In brief, peripheral blood was withdrawn from adult fasted (12 h) healthy donors that had not taken any anti-inflammatory drugs during the last 10 days, by venipuncture in heparinized tubes (16 IE heparin/ml blood). The blood was centrifuged at $4000 \times g$ for 20 min at 20 °C. Leukocyte concentrates were subjected to dextran sedimentation and centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria). Contaminating erythrocytes of pelleted neutrophils were lysed by hypotonic lysis. Neutrophils were washed twice in ice-cold PBS and finally resuspended in phosphate-buffered saline (PBS) pH 7.4 containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer) (purity > 96–97%).

For analysis of 5-LO product synthesis in whole blood as described by Pergola et al. (2008), freshly withdrawn blood from healthy adult donors was obtained by venipuncture and collected in monovettes containing 16 I.E. heparin/ml. Aliquots of 2 ml were pre-incubated with the test compounds or with vehicle (0.1%

DMSO) for 15 min at 37 °C, as indicated, and formation of 5-LO products was induced by addition of 1 μg/ml lipopolysaccharide (LPS) for 30 min at 37 °C and then 1 μM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was added. After 15 min at 37 °C, the reaction was stopped on ice and the samples were centrifuged (600 × g, 10 min, 4 °C). Aliquots of the resulting plasma (500 μl) were then mixed with 2 ml of methanol and 200 ng prostaglandin (PG)B_1 were added as internal standard. The samples were placed at −20 °C for 2 h and centrifuged again (600 × g, 15 min, 4 °C). The supernatants were collected and diluted with 2.5 ml PBS and 75 μl of 1 N HCl. Formed 5-LO metabolites were extracted and analyzed by HPLC as described for intact neutrophils below.

For analysis of 5-LO product synthesis in human neutrophils as described by Pergola et al. (2008), freshly isolated neutrophils were resuspended in 1 ml PGC buffer, preincubated for 15 min at 37 °C with test compounds or vehicle (0.1% DMSO or water), and incubated for 10 min at 37 °C with the Ca^{2+}-ionophore A23187 (2.5 μM) plus 20 μM arachidonic acid (AA). Then, the reaction was stopped on ice by addition of 1 ml of methanol, 30 μl 1 N HCl and 500 μl PBS, and 200 ng PGB_1 were added. The samples were subjected to solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA) and 5-LO products (LTB_4 and its trans-isomers, 5-H(P)ETE) were analyzed by HPLC on the basis of the internal standard PGB_1. Cysteinyl-$LTsC_4$, D_4 and E_4 were not detected (amounts were below detection limit), and oxidation products of LTB_4 were not determined.

2.3.2. 5-LO activity in cell-free assays using semi-purified 5-LO

E.coli (BL21) was transformed with pT3-5-LO plasmid, and human recombinant 5-LO protein was expressed at 30 °C as described (Fischer et al., 2003). Cells were lysed in 50 mM triethanolamine/HCl pH 8.0, 5 mM ethylen-diamine-tetracetic acid (EDTA), soybean trypsin inhibitor (60 μg/ml), 1 mM phenylmethanesulphonyl fluoride, and lysozyme (1 mg/ml), homogenized by sonication (3 × 15 s), and centrifuged at 40,000 × *g* for 20 min at 4 °C. The 40,000 × *g* supernatant was applied to an ATP-agarose column to partially purify 5-LO as described previously (Fischer et al., 2003). Aliquots of semi-purified 5-LO were immediately diluted with ice-cold PBS containing 1 mM EDTA, and 1 mM ATP was added. Samples were pre-incubated with the test compounds or vehicle (0.1% DMSO) as indicated. After 10 min at 4 °C, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$ plus 20 μM AA was added to start 5-LO product formation. The reaction was stopped after 10 min at 37 °C by addition of 1 ml ice-cold methanol, and the formed metabolites were analysed by RP-HPLC as described (Fischer et al., 2003). 5-LO products include the all-trans isomers of LTB_4 as well as 5-HPETE and its corresponding alcohol 5-HETE.

2.3.3. DPPH assay

The radical scavenging capability was assessed by measuring the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described (Blois, 1958). Briefly, 100 μl of solubilized test compounds (0.9 μg ascorbic acid, 1.2 μg L-cysteine, or 2 and 10 μg of extracts) were added to 100 μl of a solution of the stable free radical DPPH in ethanol (50 μM, corresponding to 5 nmol), buffered with acetate to pH 5.5, in a 96-well plate. The absorbance was recorded at 520 nm (Multiskan Spectrum Reader, Thermo Fisher Scientific Oy, Vantaa, Finland) after 30 min incubation under gentle shaking in the dark. Ascorbic acid and L-cysteine were used as reference compounds. All analyses were performed in triplicates.

2.3.4. Measurement of reactive oxygen species in neutrophils

Neutrophils (10^7/ml PG buffer) were preincubated with test compounds (or 0.1% DMSO as vehicle) for 15 min. Then, the peroxide-sensitive fluorescence dye 5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, 1 μg/ml) and $CaCl_2$ (1 mM) were added 2 min prior addition of phorbol myristate acetate (PMA, 0.1 μM). The fluorescence emission at 530 nm was measured after excitation at 485 nm in a thermally controlled (37 °C) NOVOstar microplate reader (BMG Labtechnologies GmbH, Offenburg, Germany).

2.3.5. Statistical analysis

Data are expressed as mean ± S.E. IC_{50} values were calculated from averaged measurements at 3–5 different concentrations of the compounds by nonlinear regression using GraphPad Prism software (San Diego, CA) one site binding competition. Statistical evaluation of the data was performed by one-way ANOVA followed by a Bonferroni or Tukey-Kramer post-hoc test for multiple comparisons respectively. A p value <0.05 (*) was considered significant.

2.4. Phytochemical screening

2.4.1. Bioassay-guided fractionation

Plectranthus zeylanicus *n*-hexane extract (230 mg) was subjected to silica gel column chromatography (Roth Kieselgel 60, 0.04–0.063 mm (230–400 mesh)). The sample was eluted with *n*-hexane, different mixtures of EtOAc in *n*-hexane (3%, 5%, 10%, 15%, 25%, 35%, 50%, 75%, 100%) and methanol successively, yielding 11 fractions. Similarly, the DCM extract (120 mg) was subjected to silica gel column chromatography. The sample was successively eluted with the same solvent mixtures mentioned above which also yielded 11 fractions. Both fractionations were repeated, the respective fractions from the two separations were combined, evaporated and the combined dry weight was measured (Table 1) and then subjected to the bioassay and LC–MS analysis.

2.4.2. Liquid chromatography coupled mass spectrometric analysis

n-Hexane and DCM extracts as well as the fractions obtained thereof were analysed on a LTQ-Orbitrap XL instrument (Thermo Fisher, San Jose, CA) with electrospray ionization. The samples were dissolved in ethyl acetate (LC–MS grade, Sigma-Aldrich, St. Louis, MO) at 1 mg/ml and diluted to 10 μg/ml. 15 μl aliquot

Table 1
Bioassay-guided separation of DCM and *n*-hexane extracts and 5-LO inhibitory activity of the fractions 1-11. Fractions F-1 to F-11 were obtained after chromatographic separations (see Methods) and the total amounts from two separate fractionations are given in mg. Isolated 5-LO was preincubated with the dissolved fractions (in DMSO) at 10 or 1 μg/ml and 5-LO activity was determined. Data are given as mean, $n=3$. n.d. = not determined.

Fraction (no.)	Total amount of *n*-hexane extract (mg)	5-LO residual activity (in %) *n*-hexane		Total amount of DCM extract (mg)	5-LO residual activity (in %) DCM	
		@ 10 μg/ml	@ 1 μg/ml		@ 10 μg/ml	@ 1 μg/ml
F-1	200	n.d.	n.d.	>1	n.d.	n.d.
F-2	1	n.d.	n.d.	48	n.d.	n.d.
F-3	1	n.d.	n.d.	>1	n.d.	n.d.
F-4	17	n.d.	n.d.	>1	n.d.	n.d.
F-5	16	n.d.	n.d.	6	n.d.	n.d.
F-6	46	53.0	n.d.	2	28.2	85.7
F-7	24	27.1	76.1	23	32.7	81.3
F-8	20	45.7	n.d.	7	n.d.	n.d.
F-9	11	**19.2**	**44.7**	5	20.2	57.5
F-10	5	**26.1**	**46.5**	7	30.2	79.2
F-11	3	46	n.d.	20	25.5	75.3

of the diluted samples were injected and separated by UPLC using a Dionex–Acclaim® RSLC 120C18 column (2.1 × 150 mm packed with 2.2 μm particles, 120 Å). Reversed phase UPLC gradient separations were performed using channel A: water (LC–MS grade, Sigma-Aldrich, St. Louis, MO, USA) with 0.1% formic acid (LC–MS, Sigma-Aldrich, St. Louis, MO) and channel D: methanol (LC–MS grade, Sigma-Aldrich, St. Louis, MO) with 0.1% formic acid as mobile phases. The gradient program was set as 0 min—100% A, 5 min—100% A, 48 min—100% D, 60 min—100% D, 60.1 min—100% A, 65.1 min—100% A and the flow rate was optimized to 0.3 ml/min.

In the Electro Spray Ionization (ESI) source, the heated capillary temperature was 275 °C and the capillary voltage and tube lens voltage were set to 48.00 V and 95.00 V respectively. The full scan and collision-induced dissociation (CID) mass spectra were generated using 30,000 and 7500 full width at half maximum (FWHM) resolutions, respectively. The full scan mass spectra were recorded in the m/z range 100–2000. CID mass spectra were obtained at different collision energies between 1 and 55 eV. The activation time was set at 30 ms with the activation parameter Q=0.25. An isolation window of 1.0 mass unit was used.

2.4.3. Molecular formula identification

Following a published method (Rasche et al., 2012), the molecular formulas were identified by isotope pattern and fragmentation tree analysis. Fragmentation trees annotate fragment peaks with molecular formulas and model fragmentation reactions through dependencies between fragment ions. The fragmentation tree that explains the data best is calculated by an optimization algorithm. The score of a tree takes into account mass deviation between peak masses and assigned molecular formulas, plausibility of molecular formulas, intensity of explained peaks, and whether losses are common.

2.4.4. Gas chromatography coupled mass spectrometric analysis

The fractions of interest (1 mg/ml in ethyl acetate) were analyzed on a gas chromatograph HP6890 (Agilent, CA) connected to a MS02 mass spectrometer from Micromass (Waters, Manchester, UK) with EI 70 eV using ZB5ms column (30 m × 0.25 mm, 0.25 μm film thickness; Phenomenex, CA). The carrier gas was helium at the flow rate of 1 ml/min. The injector temperature was kept at 250 °C and the temperature program was set as 100 °C (2 min), 15 °C /min to 200 °C, 5 °C/min to 305 °C (20 min). The available standards [stigmasterol (Fluka), β-sitosterol (Aldrich) and phytol (Aldrich)] were also analysed under the same GC–MS conditions.

3. Results

3.1. Evaluation of 5-LO inhibitory activities

To investigate suppression of 5-LO activity in the cell, A23187-stimulated human neutrophils (Werz and Steinhilber, 2005) were used. In the course of a screening approach of extracts of diverse native plants from Sri Lanka used as anti-inflammatory remedies to interfere with 5-LO activity, we identified lipophilic extracts of *Plectranthus zeylanicus* as potential hits. Thus, *Plectranthus zeylanicus* extracts (100 μg/ml) that were prepared by using *n*-hexane or DCM as solvent strongly inhibited 5-LO activity in A23187-stimulated neutrophils (inhibition > 85%), whereas extracts obtained by the use of hydrophilic solvents, that is, water or methanol were comparably ineffective and reduced 5-LO activity only by < 25% at a concentration of 100 μg/ml (Fig. 1A). More detailed concentration-response studies using this cell-based assay revealed IC_{50} values of 6.6 and 12 μg/ml for *Plectranthus zeylanicus* extracts prepared with *n*-hexane and DCM, respectively (Fig. 1B). The synthetic reference inhibitor zileuton (approved as anti-asthmatic drug) (Israel et al., 1990) blocked 5-LO activity with IC_{50}=0.13 μg/ml (corresponding to 0.55 μM). Note that unspecific detrimental effects of the extracts on the viability of neutrophils can be excluded based on the ability of the cells to prevent trypan blue uptake in the presence of 10 or 100 μg/ml (not shown).

Suppression of 5-LO product synthesis in the cell may be caused by diverse mechanisms, others than interference with the 5-LO enzyme activity, including inhibiting of substrate supply,

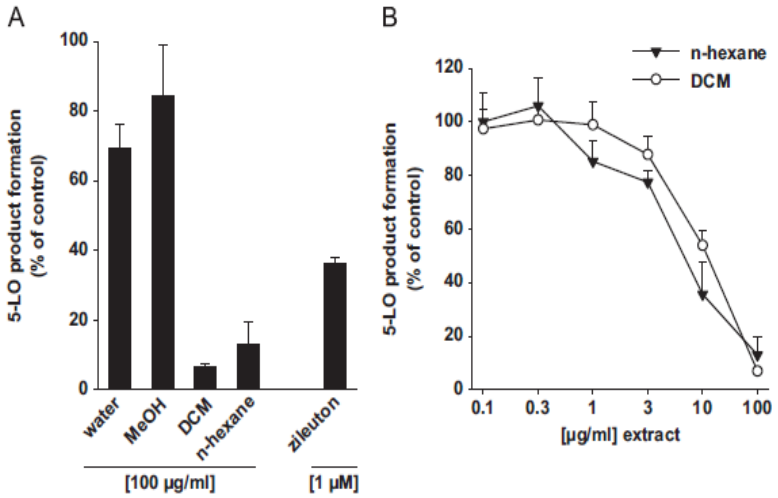

Fig. 1. Inhibition of 5-LO activity in intact human neutrophils. (A) Comparison of the 5-LO inhibitory activity of *Plectranthus zeylanicus* extracts (100 μg/ml, each) prepared by the use of water, methanol (MeOH), dichloromethane (DCM) or *n*-hexane as solvent; zileuton (1 μM) was used as reference compound. (B) Concentration-response curves for DCM and *n*-hexane extracts. Freshly isolated human neutrophils (5×10^6/ml) PGC buffer were preincubated with the indicated concentrations of *Plectranthus zeylanicus* extracts or vehicle (0.1% DMSO) for 15 min at 37 °C. 5-LO product formation was induced by addition of 2.5 μM A23187 plus 20 μM AA, and after 10 min, the reaction was stopped by addition of 1 ml methanol and 5-LO products were analyzed by HPLC. Data are expressed as percentage of vehicle control (100%), means ± S.E., n=3.

blockade of FLAP, or loss of cell viability. In order to investigate whether or not the extracts directly inhibit 5-LO activity, we applied a cell-free assay using isolated human recombinant 5-LO. As shown in Fig. 2, the *n*-hexane and DCM extracts of *Plectranthus zeylanicus* caused concentration-dependent inhibition of 5-LO with IC_{50} = 1.2 and 0.7 μg/ml, respectively. For zileuton, the IC_{50} value was determined at 0.11 μg/ml (not shown), implying comparably marked 5-LO inhibitory potencies of the *Plectranthus zeylanicus* extracts.

Finally, 5-LO inhibition by *Plectranthus zeylanicus* extracts was investigated in LPS/fMLP-stimulated human whole blood, a robust and biological relevant test system that includes several variables (e.g., plasma protein binding) which may impede the efficiency and thus, allows for judging the pharmacological potential of a given 5-LO inhibitor. Both, the *n*-hexane and the DCM extract suppressed 5-LO product formation in human whole blood by approx. 40% at 100 μg/ml (Fig. 2B). Higher extract concentrations could not be tested due to solubility issues. For zileuton, an IC_{50} of

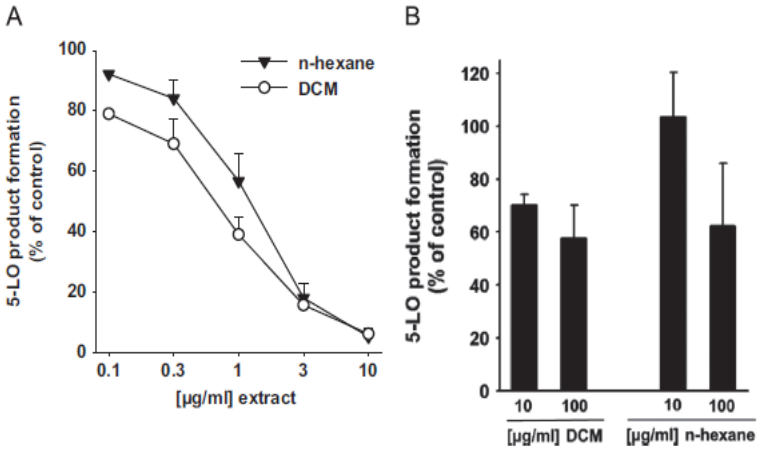

Fig. 2. Inhibition of isolated 5-LO in a cell-free assay and suppression of 5-LO activity in human whole blood. (A) Inhibition of 5-LO activity in a cell-free assay. Partially purified recombinant 5-LO (0.5 μg/ml) was incubated with dichloromethane (DCM) and *n*-hexane extracts of *Plectranthus zeylanicus* or vehicle (0.1% DMSO) at 4 °C for 15 min. Samples were prewarmed for 30 s at 37 °C, 2 mM $CaCl_2$ and 20 μM AA were added, and 5-LO product formation was determined after 10 min. Data are expressed as percentage of control (100%), means ± S.E., n = 3. (B) Inhibition of 5-LO activity in whole blood. Freshly withdrawn human whole blood was preincubated with the test compounds or with vehicle (0.1% DMSO) for 15 min at 37 °C, as indicated, and 1 μg/ml LPS was added. After 30 min at 37 °C, 1 μM fMLP was added and after another 15 min the reaction was stopped and 5-LO product synthesis was analysed. Data are expressed as percentage of vehicle control (100%), means ± S.E., n = 3.

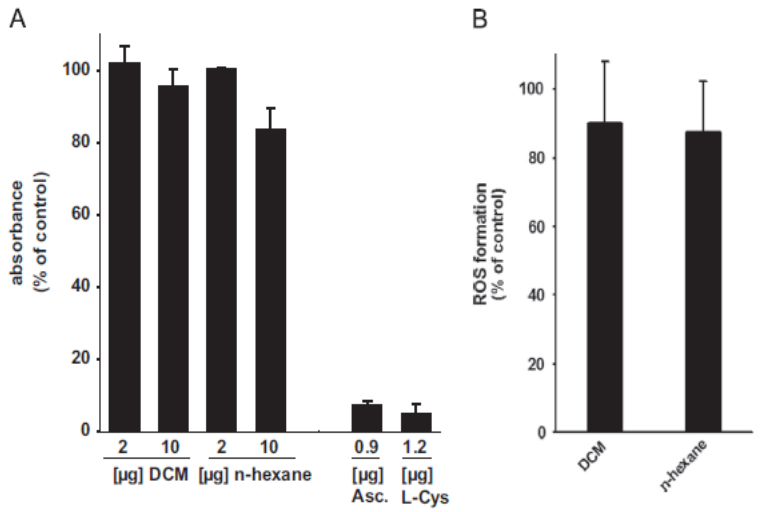

Fig. 3. Analysis of radical scavenging activities and cellular formation of reactive oxygen species. (A) Radical scavenging activities. *Plectranthus zeylanicus* extracts were incubated with 5 nmol DPPH for 30 min at RT and the absorbance was measured at 520 nm. Ascorbic acid and L-cysteine were used as controls. Values are given as percentage of control (100%) mean ± S.E., n = 3. (B) Analysis of cellular ROS formation. Neutrophils were pre-incubated with *Plectranthus zeylanicus* extracts for 15 min, loaded with the fluorescent dye DCF-DA and stimulated with 0.1 μM PMA. The relative increase in fluorescence was determined after at 37 °C after 360 s. Data (mean ± S.E., n = 3) are expressed as–fold increase (t = 0 and t = 360 s).

0.9 μM was obtained that agrees with literature data (Carter et al., 1991).

3.2. Evaluation of radical scavenging properties and suppression of ROS formation in neutrophils

The majority of natural products from plant origin that suppress 5-LO activity may confer their inhibitory action by unselective antioxidant reactions as they reduce the active-site iron, decompose 5-LO-activating lipid hydroperoxides, or scavenge intermediate fatty acid radicals within LT synthesis (Werz, 2007). To investigate whether such unselective antioxidant properties may account also for 5-LO inhibition by *Plectranthus zeylanicus* extracts, we assessed radical scavenging properties using the cell-free DPPH assay. In contrast to the antioxidants ascorbic acid or L-cysteine (used a reference compounds) the *n*-hexane or DCM extracts of *Plectranthus zeylanicus* were not able to significantly reduce radical formation up to a final concentration of 50 μg/ml (corresponding to 10 μg/200 ml in the assay, Fig. 3A), suggesting that 5-LO inhibition is not mediated by a redox-based mechanism.

In addition, we studied the ability of the extracts to prevent cellular ROS formation in neutrophils stimulated with fMLP. Diphenyleneiodonium chloride (DPI, 10 μM) was used as reference inhibitor that completely abolished ROS formation (not shown), as

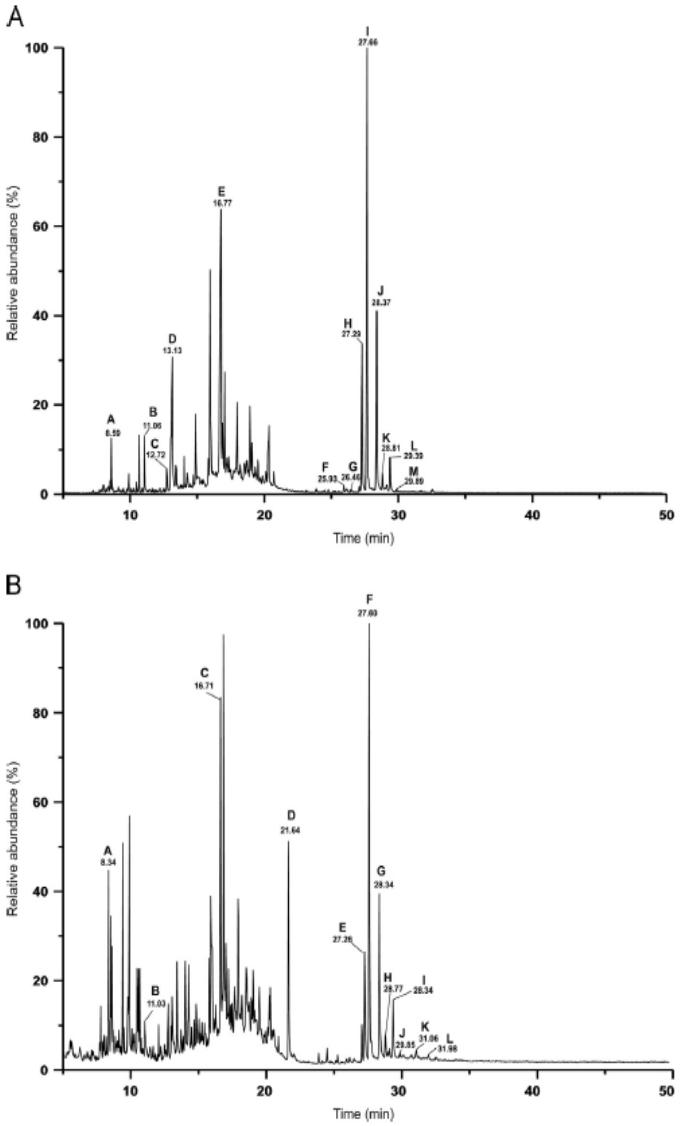

Fig. 4. Identification of constituents of bioactive fractions of the *n*-hexane extract of *Plectranthus zeylanicus* by GC–MS. (A) Total ion chromatograph of F-9 of the *n*-hexane extract of *Plectranthus zeylanicus* and its identified compounds. A: Eudesm-7(11)-en-4-ol, B: hexadecanoic acid, C: phytol, D: 9,12,15-octadecatrienoic acid, E: callitrisic acid, F: cholest-5-en-3β-ol, G: ergosta-5,22-dien-3β-ol, H: campesterol, I: stigmasterol, J: β-sitosterol, K: β-amyrin, L: α-amyrin, M: stigmast-4-en-3-one. (B) Total ion chromatograph of F-10 of the hexane extract of *Plectranthus zeylanicus* and its identified compounds. A: 4-[(1E)-3-hydroxy-1-butenyl]-3,5,5-trimethyl-2-cyclohexen-1-one, B: hexadecanoic acid, C: callitrisic acid, D: 13-docosenamide, E: campesterol, F: stigmasterol, G: β-sitosterol, H: β-amyrin, I: α-amyrin, J: stigmast-4-en-3-one, K: 4-methylstigmast-22-en-3-one, L: stigmastane-3,6-dione.

expected. In accordance with the DPPH assay, neither the *n*-hexane nor the DCM extract of *Plectranthus zeylanicus* caused significant inhibition of ROS formation (Fig. 3B).

3.3. Bioassay-guided separation of the Plectranthus zeylanicus extracts

We attempted to get more insights into the identity of the ingredients and composition of the extracts that might be responsible for the potent inhibition of 5-LO. Hence, we separated the *n*-hexane and DCM extracts by liquid column chromatography using ethyl acetate, *n*-hexane, and methanol as solvents into 11 fractions that were analyzed for inhibition of isolated 5-LO in the cell-free assay at 1 and 10 μg/ml, each. Out of the 11 fractions (F) of the *n*-hexane extract, F-6 (25% EtOAc in *n*-hexane), F-7 (35% EtOAc in *n*-hexane), F-8 (50% EtOAc in *n*-hexane), F-9 (75% EtOAc in *n*-hexane), F-10 (EtOAc) and F-11 (MeOH) at a concentration of 10 μg/ml inhibited 5-LO activity by > 50%, and only F-9 and F-10 were active at 1 μg/ml (> 50% 5-LO inhibition) (Table 1). For the DCM extract, F-6 (25% EtOAc in *n*-hexane), F-7(35% EtOAc in *n*-hexane), F-9 (75% EtOAc in *n*-hexane), F-10 (EtOAc) and F-11 (MeOH) were active at 10 μg/ml concentration in the 5-LO cell-free assay, but none of these fractions showed significant 5-LO inhibitory activity at 1 μg/ml.

3.4. Characterization of constituents of bioactive fractions of the n-hexane extract of Plectranthus zeylanicus

An UPLC system coupled to LTQ Orbitrap XL instrument and GC–MS have been employed as sole techniques for structural characterization of secondary metabolites. The accurate mass measurements of adduct ions by the Orbitrap instrument enabled the determination of molecular composition within 1–5 ppm mass errors and database searching of exact masses for possible relevant secondary metabolites. MS/MS data has provided a powerful tool to de-replicate possible structures. To obtain better overview, the fragments obtained from the MS/MS experiments were further analyzed by computer-assisted algorithms to yield hypothetical fragmentation trees which allow the assignment of specific relevant fragments and fragmentation pathways.

3.4.1. GC–MS analysis

Due to the high 5-LO inhibitory activity of F-9 and F-10 from the *n*-hexane extract of *Plectranthus zeylanicus*, these fractions were subjected to a phytochemical screening by (I) GC–MS and (II) UPLC–MS. The GC–MS analysis of F-9 revealed 13 components, identified by comparison of their experimental mass spectrum with those recorded in the NIST MS Search 2.0 and Adams mass

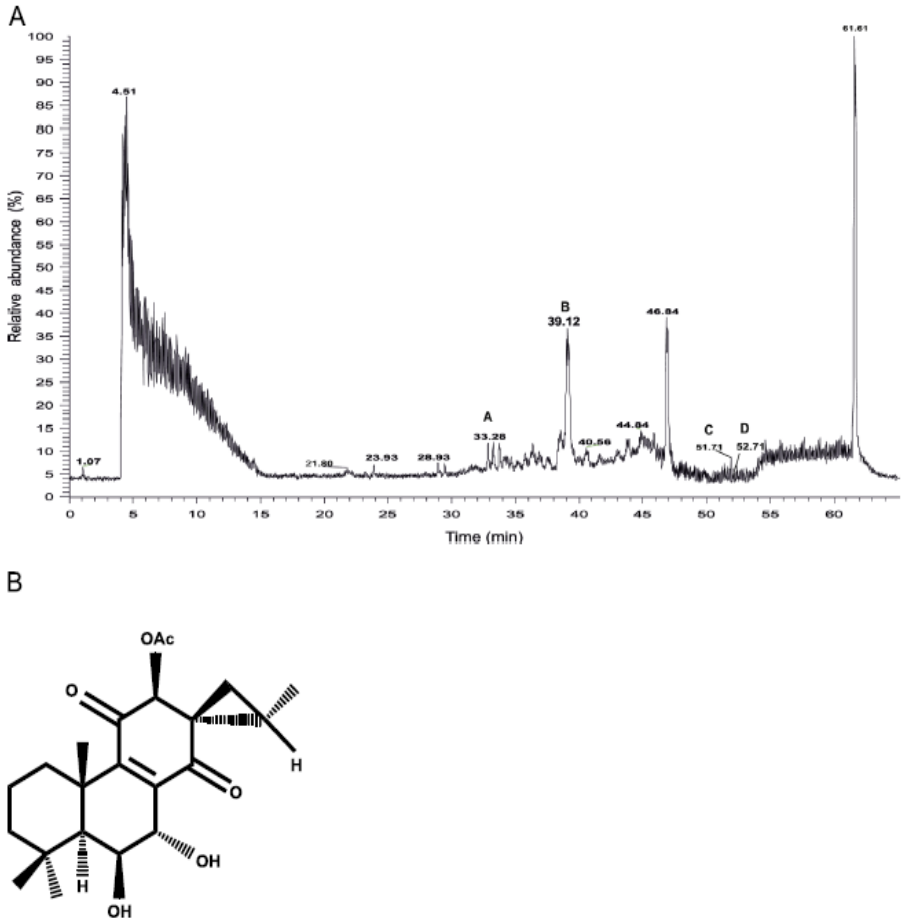

Fig. 5. Identification of constituents of bioactive fractions of the *n*-hexane extract of *Plectranthus zeylanicus* by UPLC–MS. (A) Total ion chromatogram of F-9 of the hexane extract of *Plectranthus zeylanicus* and its identified compounds. A: cinncassiol A/cinncassiol C3, B: coleone P, C: stigmasterol, D: stigmasterol-5,22,25-trien-3-β-ol. (B) Chemical structure of coleone P.

spectrum libraries as well as by comparison with the respective standards. Among these components, ubiquitously occurring fatty acids (hexadecanoic acid, 9,12,15-octadecatrienoic acid), phytol and eudesm-7(11)-en-4-ol, as well as several frequent phytosterols and derivatives (i.e., cholest-5-en-3β-ol, ergosta-5,22-dien-3β-ol, campesterol, stigmasterol, β-sitosterol, stigmast-4-en-3-one) were detected. However, also α- and β-amyrin and callitrisic acid, a quite uncommon diterpenoic phenanthrenecarboxylic acid, were present (Fig. 4A).

GC–MS analysis of F-10 revealed 12 compounds; many of them were the same as in F-9, including phytosterol derivatives, hexadecanoic acid, amyrins, and callitrisic acid (Fig. 4B). In addition, 4-[(1E)-3-hydroxy-1-butenyl]-3,5,5-trimethyl-2-cyclohexen-1-one and 13-docosenamide were detected in F-10.

3.4.2. UPLC–MS analysis

Analysis of F-9 by UPLC–MS revealed two uncommon compounds (denoted as compound A and B, Fig. 5A) and two common phytosterol derivatives that were detected in the TIC. In F-10, only compound B yielded a substantial peak reflecting significant amounts present in this fraction (not shown). In addition, a small peak corresponding to compound A was apparent in the TIC of F-10.

The accurate mass measurements of compound B using the Orbitrap instrument and the search in the METLIN database suggested the presence of coleone P ($C_{22}H_{30}O_6$, Fig. 5B) and its sodium salt at *m/z* 391.21255 and 413.19431, respectively, in both F-9 and F-10. The identity of coleone P was further deduced by its fragmentation pattern, which agrees with literature data (Arihara et al., 1975). The collision-induced dissociation spectra of the protonated adduct at different collision energies are given in Fig. S1. The computed hypothetical fragmentation trees for both protonated and sodium adducts, which annotate fragment masses with molecular formulas and show the dependencies between the fragments through neutral losses, are given in Figs. S2 and S3.

In addition, the peak at *m/z* 383.20648 which corresponds to compound A, fits well with the molecular formula $C_{20}H_{30}O_7$ (mass accuracy of 0.131 ppm) suggesting the presence of cinncassiol A/cinncassiol C3, a diterpenoid which has been isolated from the family Lauraceae. The computed hypothetical fragmentation tree for the protonated adduct is given in Fig. S4. Unfortunately, the unavailability of authentic standards of compound A and B has hindered the UPLC–MS confirmation of the respective structures.

4. Discussion

Here we attempted to (I) rationalize and validate the traditional use of the medicinal plant *Plectranthus zeylanicus* as anti-inflammatory remedy by analysis of its ability to interfere with 5-LO activity and (II), to identify relevant constituents of the bioactive fractions. In fact, lipophilic extracts of *Plectranthus zeylanicus* using *n*-hexane or DCM as solvents caused direct and potent inhibition of human 5-LO and suppressed the biosynthesis 5-LO products in isolated human neutrophils and even in human whole blood. Notably, these extracts exhibited no significant radical scavenging or antioxidant activities in cell-free (DPPH assay) or cell-based (ROS generation in neutrophils) test systems, and failed to reduce neutrophil viability. Hence, we conclude that lipophilic *Plectranthus zeylanicus* extracts contain nonredox-related principles that specifically interact with 5-LO supporting an anti-inflammatory potential. Instead of performing extensive chromatographic separations and isolation procedures aiming to reveal potential bioactive constituents by applying traditional phytochemical analysis, rapid and convenient chromatographic/MS approaches were employed for compound identification. Provision of these so far unavailable compounds (i.e., coleone P) in large scale will allow for future comprehensive bioactivity studies and reveal whether or not they inhibit 5-LO.

Since the *Plectranthus zeylanicus* is widely used in traditional medicine in Sri Lanka and South India to alleviate the pathological conditions caused by inflammation (Ayurveda Pharmacopeia, 1979; Jayaweera, 1982), we focused on inhibition of 5-LO as potential underlying mode of action. In fact, 5-LO as key enzyme in the biosynthesis of the pro-inflammatory LTs (Radmark et al., 2007) is explored as drug target for the intervention with asthma, allergic rhinitis, various autoimmune diseases, cardiovascular disease, and many other inflammatory disorders (Peters-Golden and Henderson, 2007). Direct 5-LO inhibitors (of synthetic or natural origin) are essentially categorized as (I) redox-type inhibitors that interfere with the redox cycle of the 5-LO active-site iron, (II) iron ligand-type inhibitors that chelate the active-site iron, and (III) nonredox-type inhibitors that compete with fatty acid substrate and/or activating fatty acid hydroperoxides or undefined modes of action (Werz and Steinhilber, 2005; Pergola and Werz, 2010). Starting from early 1980s until today, several hundred plants and their extracts and/or specific secondary metabolites thereof have been reported that are capable of suppressing the biosynthesis of 5-LO products (for review see: Schneider and Bucar, 2005). Such interference with 5-LO activity is considered as basis for the anti-inflammatory features of the respective plants (and medical preparations thereof) in folk medicine. However, many of these investigations lacked sufficient and detailed experimentation and the 5-LO inhibitory potencies of the extracts often turned out to be comparably low. Thus, lipophilic extracts of well-recognized anti-inflammatory medicinal plants such as *Tripterygium wilfordii*, *Urtica dioica*, *Zingiber officinale* or *Harpagophytum procumbens* blocked cellular 5-LO activity with relatively high IC_{50} values of approx. 20–80 μg/ml (Schneider and Bucar, 2005). Also, the reviewed literature (Schneider and Bucar, 2005) reveals 5-LO inhibitory activity of lipophilic extracts of various other medicinal plants with IC_{50} values in the rough average range of 15–50 μg/ml. For example, even for extracts of the gum resin of *Boswellia serrata*, which is considered as potent 5-LO inhibiting natural product and thus frequently used as anti-inflammatory remedy (Ammon, 2006), IC_{50} values for 5-LO in neutrophils of 8.4–30 μg/ml were determined (Ammon et al., 1993; Wildfeuer et al., 1998). In direct comparison to these potencies, the results obtained with the *n*-hexane and DCM extracts of *Plectranthus zeylanicus* with IC_{50} of 0.7–12 μg/ml are remarkable and suggest a high pharmacological potential of *Plectranthus zeylanicus* for intervention with 5-LO-related disorders.

The active constituents of various plant preparations responsible for 5-LO inhibition were often proposed to be flavonoids, polyphenols, coumarins, and hydroquinones that all possess antioxidant activities, and this was correlated to enzyme inhibition (Werz, 2007). In contrast, for the 5-LO inhibitory *Plectranthus zeylanicus* extracts, we could not observe significant antioxidant properties, suggesting that the respective bioactive constituents mediate 5-LO inhibition by specific interference in a nonredox-dependent manner. Note that the potencies of the *Plectranthus zeylanicus* extracts were more pronounced (about 8-fold) when inhibition of isolated 5-LO was studied as compared to cellular 5-LO suppression. Possibly, the active constituents fail to reach equivalent concentrations inside the cell (poor permeation) or cellular factors may impair 5-LO inhibition by competition, unspecific protein binding or degradation of the constituents.

MS in combination with the hyphenated chromatographic techniques is emerging as a powerful tool in small molecule identification. The resolving power of the chromatography has tremendously enhanced by the introduction of UPLC (Eugster et al., 2011), while the Orbitrap mass accuracy permits a straightforward determination of the molecular composition of parent and

fragment ions (Perry et al., 2008). Although in its infancy, computational methods for the automated analysis of tandem MS data are now developing as potential utensils in characterization of small molecules. Moreover, the GC–MS analysis followed by the comparison of a measured spectrum to reference spectra in databases has been well established as an effective identification method of volatile natural products since many years.

These outstanding developments in the areas of separation methods and spectrometric techniques afford a rapid identification and characterization of secondary metabolites without the necessity of isolation and purification, while the detailed information about their metabolic profiles can be obtained with a minimal amount of material. Utilizing efficient chromatographic and MS techniques, we have performed an initial study on the putative bioactive constituents in a restricted fraction of the *n*-hexane extract of *Plectranthus zeylanicus* after chromatographic separation. We have used tandem mass spectrometry and the accurate mass measurements data to tentatively identify the compound in the active fractions. Our approach is novel in the fact that allows for fast data de-replication and will give the researchers a hint on the class of compounds. The amounts of compounds we are able to assay using MS/MS experiments is typically much smaller and it can be successfully applied to mixtures. For NMR measurements more sample will be needed, therefore large scale extraction and purification procedures are required, thus the pure compound isolation was beyond the scope of this initial phytochemical study.

None of the studies conducted so far on this plant has correlated the occurrence of a specific secondary metabolite to bioactivity, apart from a study which assigned caffeic acid and coumaric acid to antioxidant activity of the plant extract (Rasineni et al., 2008). Therefore, our study provides new insights towards phytochemicals of *Plectranthus zeylanicus*, paving the way for more detailed analysis in future. Among the identified compounds in the most active fractions, some have been proposed to possess in vitro and in vivo anti-inflammatory properties (Kweifio-Okai and Macrides, 1992; Prieto-Garcia et al., 2006).

Among the identified constituent in the active fractions of the *n*-hexane extract of *Plectranthus zeylanicus*, coleone P appears to be of particular interest. The genus *Plectranthus* is rich in coleone-type diterpenoids which exert several biological activities. For example, coleone U and C showed strong antiproliferative activities against human cancer cell lines, respectively (Marques et al., 2002; Xing et al., 2008), and for coleone U, antimicrobial activities were observed (Wellsow et al., 2006). Coleone P has been isolated from the *Plectranthus* species *Plectranthus caninus*, and our spectral analysis proposes the presence of this compound in the active fractions F-9 and F-10 of the *Plectranthus zeylanicus n*-hexane extract. However, the absence of authentic standards of this compound impedes the detailed studies on its anti-inflammatory properties. Besides coleone P, cinncassiol A/C3 as well as callitrisic acid were identified for which the knowledge regarding bioactivities is rare. Furthermore, the pentacyclic triterpenes α- and β-amyrin which have displayed anti-inflammatory activity in in vitro and in vivo models are present in the active fractions (Kweifio-Okai and Macrides, 1992).

Although it is reasonable to correlate the 5-LO inhibitory activity of *Plectranthus zeylanicus* to the presence of the identified compounds supported also on literature reports, there are several unknown compounds that are not in any database which might also contribute to the bioactivity. Therefore, we expect to expand our research towards the identification of these compounds in the active fractions in future studies. The recently introduced fragmentation trees for the automated comparison of fragmentation patterns of small molecules (Rasche et al., 2012) will be used for this purpose. The computed fragmentation trees (employed in this study) for compound identification has provided a good explanation of our observed data, and hence, could be a valuable tool for the identification of the unknown constituents. Once the identification is completed, we hope either to synthesize or conduct a large scale extraction (depending on the availability of plant material) to isolate such compounds in sufficient amounts in order to perform discrete analysis of their bioactivities, in particular with respect to inhibition of 5-LO.

5. Conclusion

By demonstrating potent inhibition of 5-LO activity by lipophilic extracts of *Plectranthus zeylanicus* in different biological test systems, our results may explain the traditional use of *Plectranthus zeylanicus* in Sri Lanka and South India for the treatment of inflammatory conditions. We conclude that even though 5-LO is a redox-sensitive oxygenase, the potent inhibition of 5-LO activity is not related to unselective antioxidant properties but rather due to more discrete and direct interference of the respective constituents with the enzyme. The phytochemical analysis of the bioactive fractions by MS techniques led to the identification of coleone P for the first time in this plant, along with other thus far unknown or potentially interesting constituents for which anti-inflammatory activity has been proposed. Provision of these candidate compounds in large scale may afford to analyze in detail whether or not they interfere with 5-LO and thus, constitute active principles rationalizing the anti-inflammatory use of *Plectranthus zeylanicus* in folk medicine.

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Appendix A. Supplementrary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2013.11.004.

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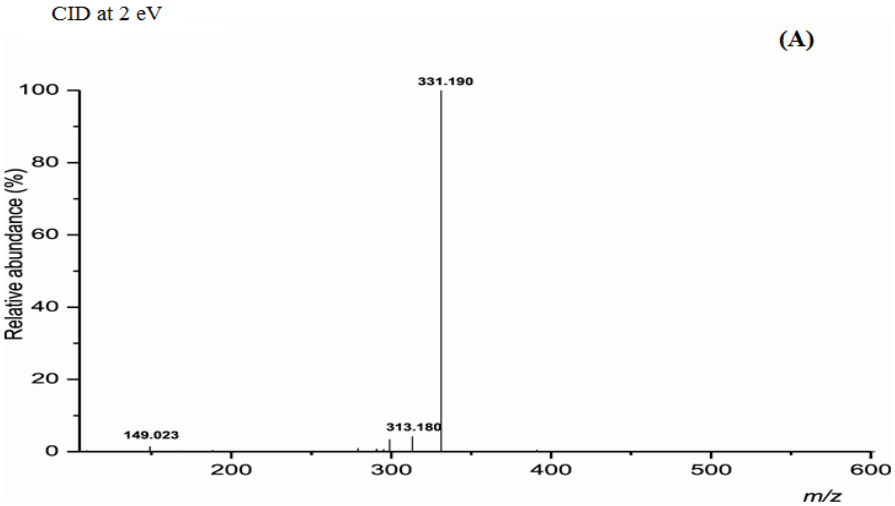
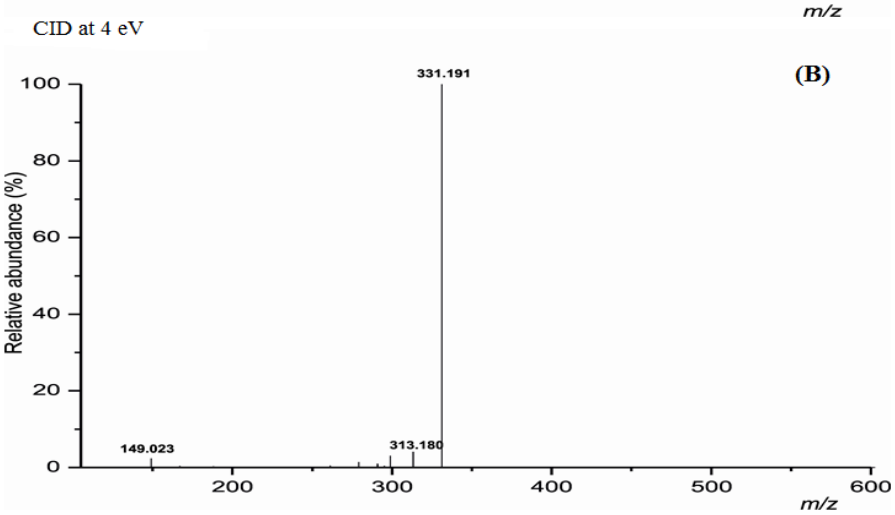
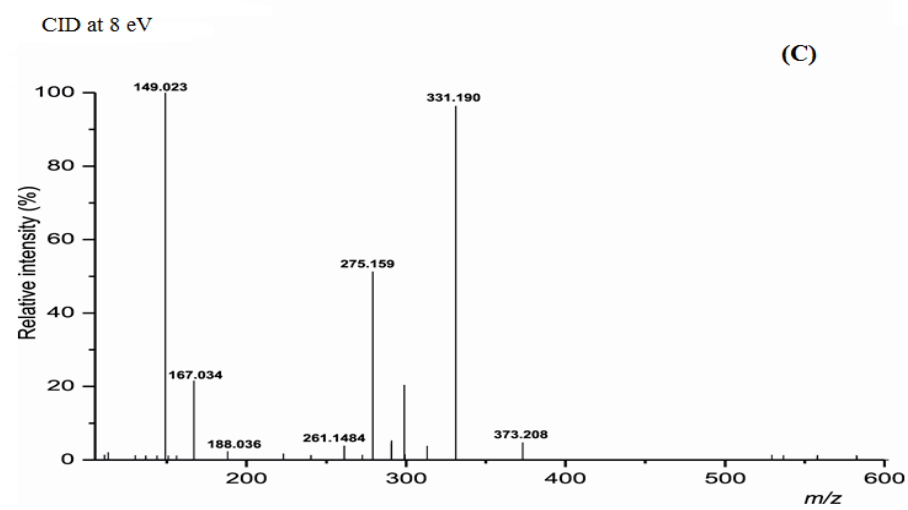
Supplementary material – 1







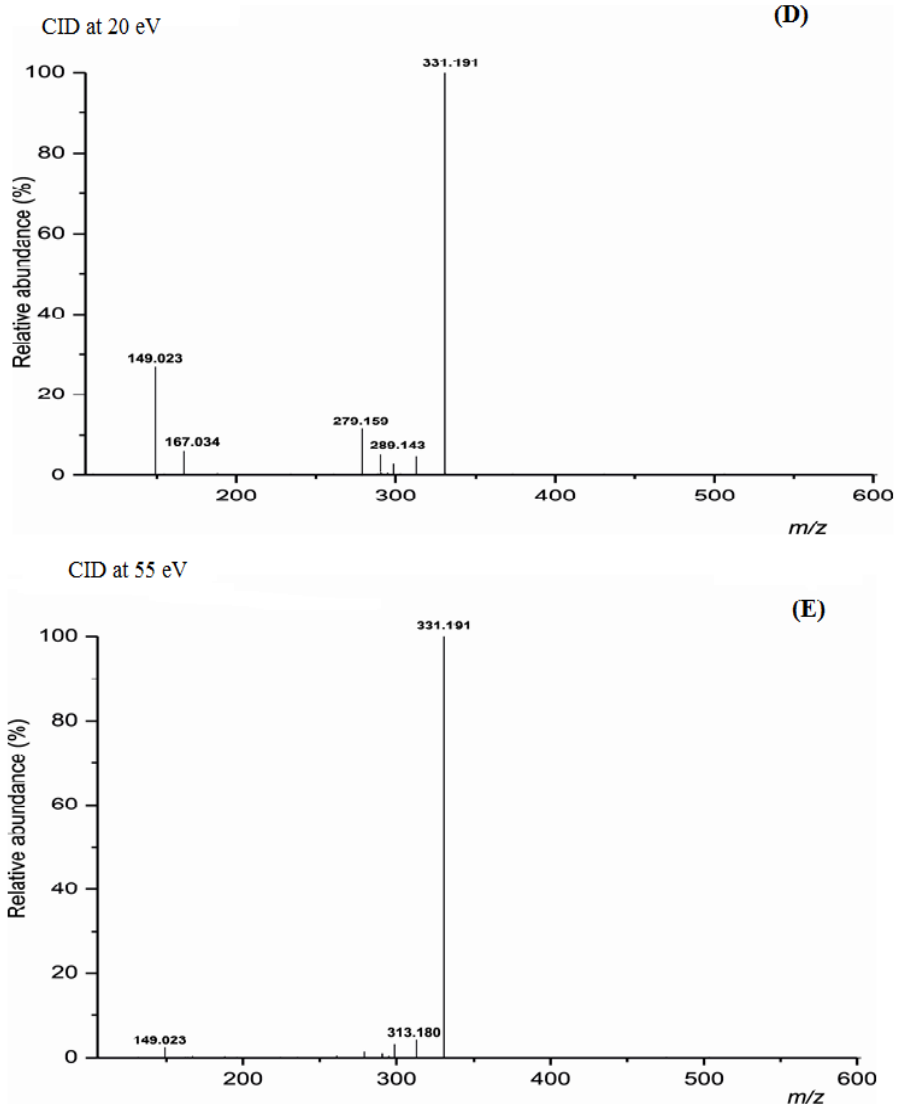


Fig. S-1: Collision-induced spectra of the protonated adduct of Coleone P at different collision energies, **(A)** 2 eV , **(B)** 4 eV, **(C)** 8 eV, **(D)** 20 eV and **(E)** 55 eV

Supplementary material -2

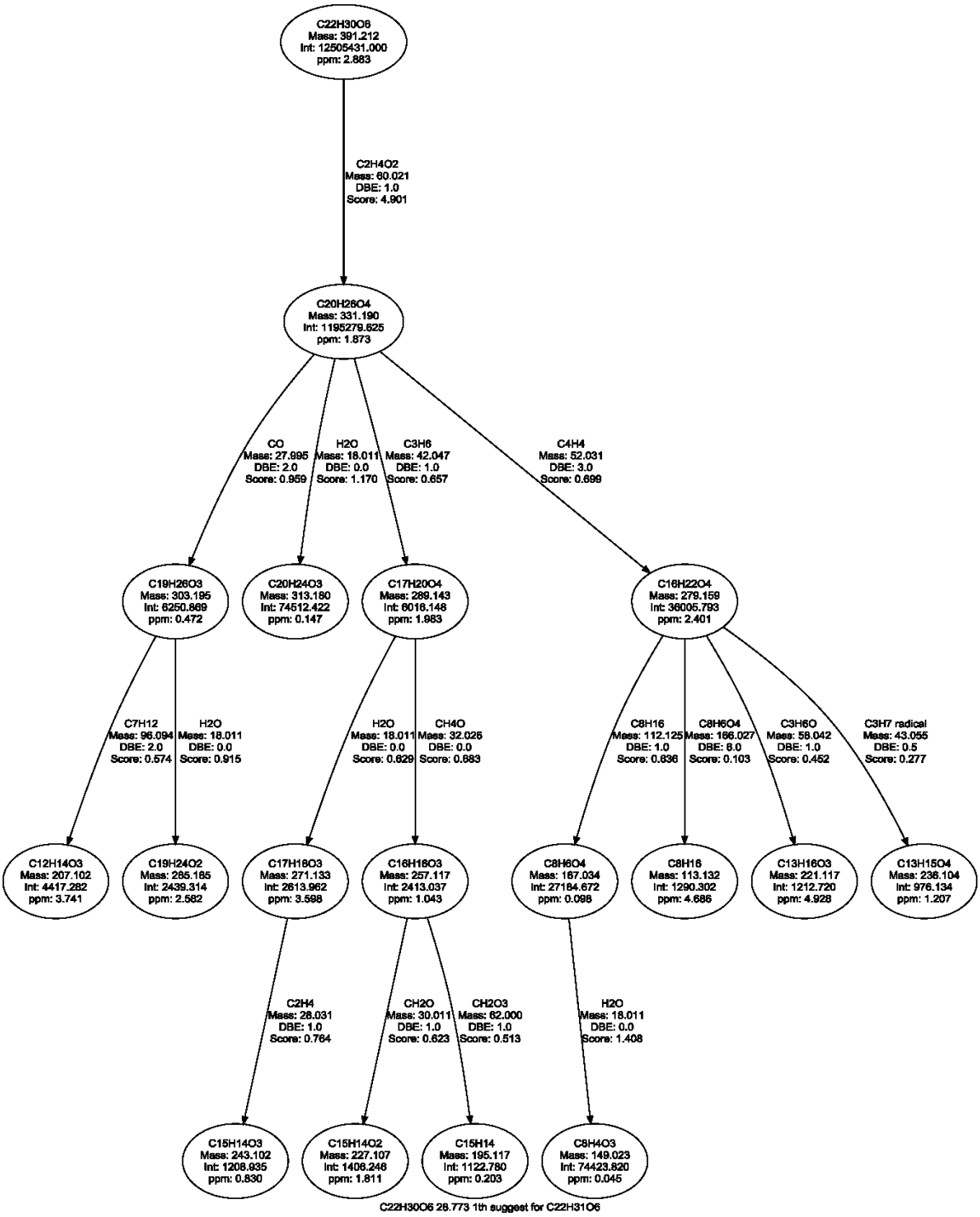


Fig. S-2: Hypothetical fragmentation tree for the protonated adduct of Coleone P

Supplementary material -3

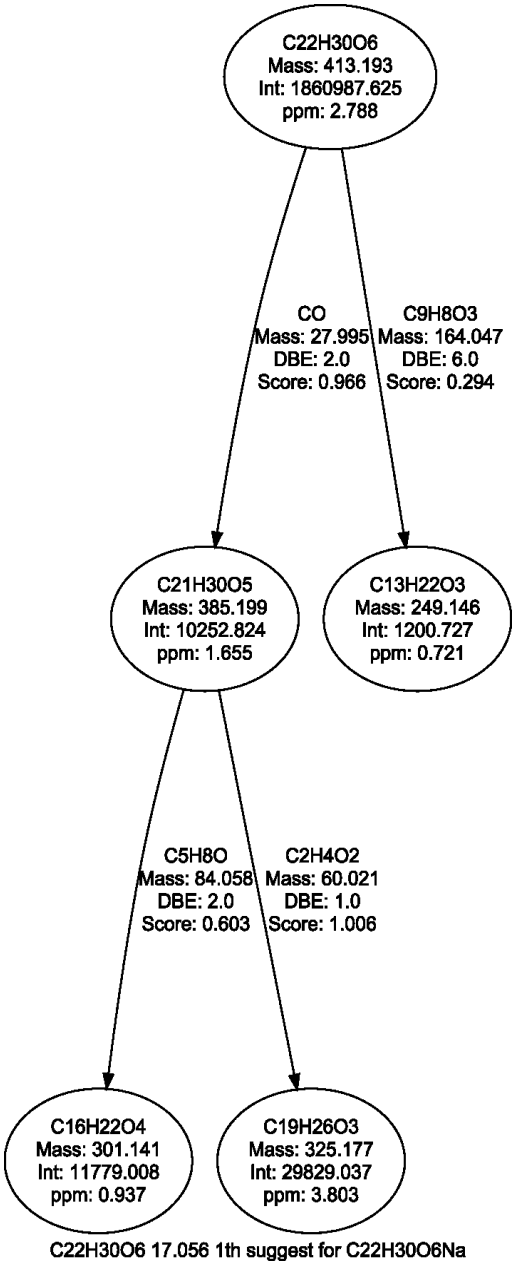


Fig. S-3: Hypothetical fragmentation tree for the sodium adduct of Coleone P

Supplementary material -4

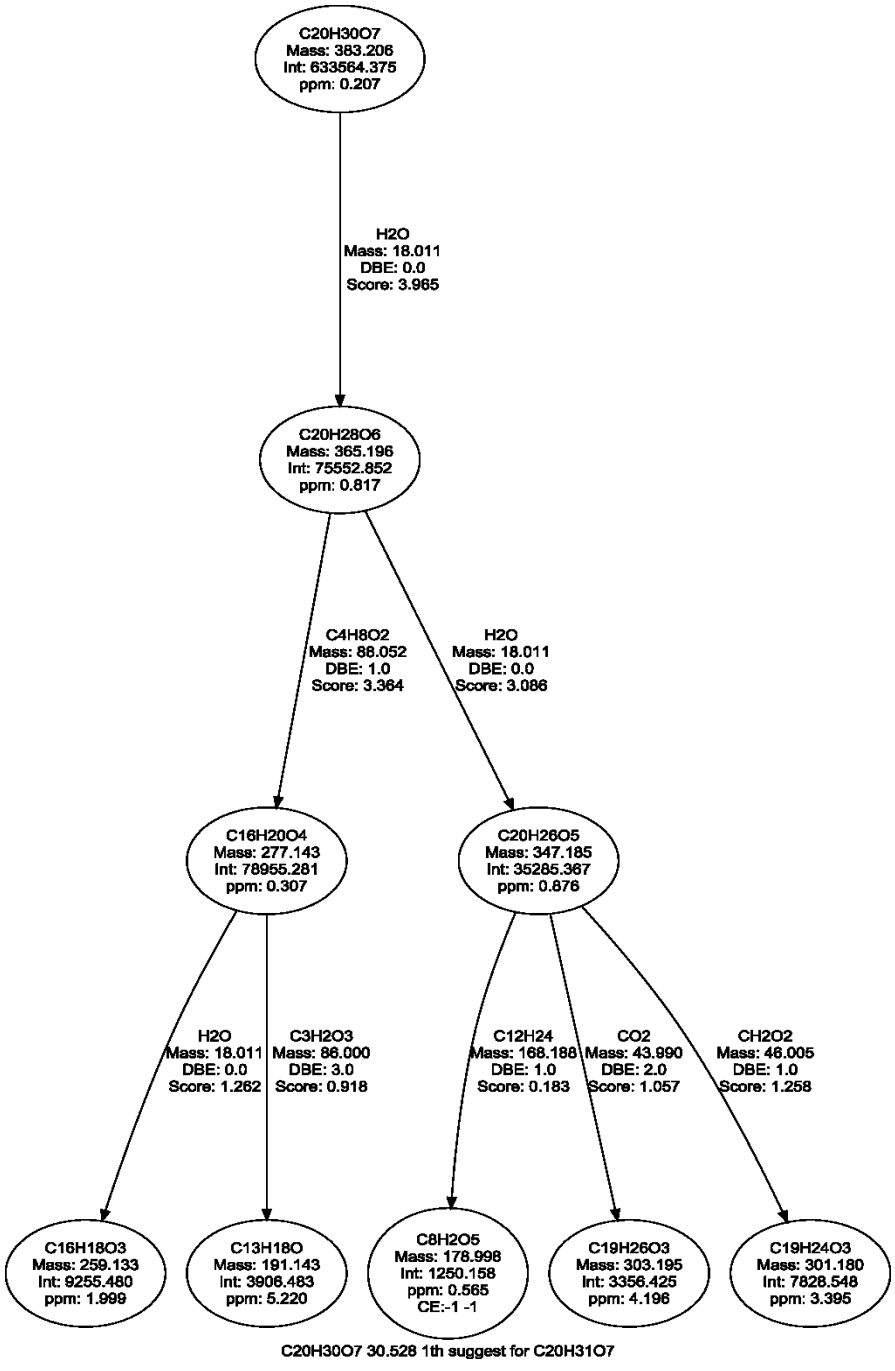


Fig. S-4: Hypothetical fragmentation tree for the protonated adduct of Cinncassiol A / Cinncassiol C3

Chapter 4

Manuscript-III

Munronia pinnata (Wall.) Theob.: Unveiling Phytochemistry & Dual Inhibition of 5-lipoxygenase and Microsomal Prostaglandin E_2 Synthase (mPGES)-1

Mayuri Napagoda, Jana Gerstmeier, Andreas Koeberle, Sandra Wesely, Sven Popella, Sybille Lorenz, Kerstin Scheubert, Sebastian Boecker, Aleš Svatoš & Oliver Werz

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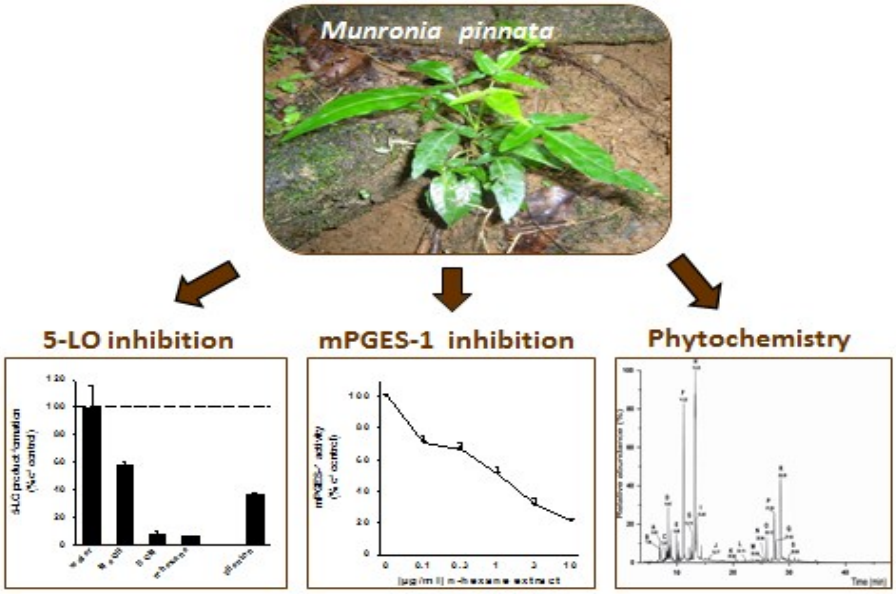

Abstract

Ethnopharmacological relevance: Preparations from *Munronia pinnata* (Wall.) Theob are extensively used in traditional medicine in Sri Lanka for the treatment of inflammatory conditions. However, neither the pharmacological features nor the phytochemistry of this plant are explored in order to understand and rationalize the reported ethnobotanical significance. As 5-lipoxygenase (5-LO) and microsomal prostaglandin E_2 synthase (mPGES)-1 are crucial enzymes in inflammatory disorders, we evaluated their inhibition by *M. pinnata* extracts and studied the chemical profile of the plant for the identification of relevant constituents.

Materials and Methods: Cell-free and cell-based assays were employed in order to investigate the suppression of 5-LO and mPGES-1 activity. Cell viability, radical scavenger activities, and inhibition of reactive oxygen species formation (ROS) in neutrophils were studied to assess cytotoxic or antioxidant effects. Gas or liquid chromatography coupled to mass spectrometric analysis enabled the characterization of secondary metabolites.

Results: The *n*-hexane extract of *M. pinnata* efficiently suppressed 5-LO activity in stimulated human neutrophils (IC_{50} = 8.7 µg/ml) and potently inhibited isolated human recombinant 5-LO (IC_{50} = 0.48 µg/ml) and mPGES-1 (IC_{50} = 1.0 µg/ml). In contrast, no significant radical scavenging activity or suppression of ROS formation was observed, and neutrophil viability was unaffected. The phytochemistry of the plant was unveiled for the first time and phytosterols, fatty acids, sesquiterpenes and several other types of secondary metabolites were identified.

Conclusions: Together, potent inhibition of 5-LO and mPGES-1 activity, without concomitant antioxidant activity and cytotoxic effects, rationalizes the ethnopharmacological use of *M. pinnata* as anti-inflammatory remedy. Modern chromatographic/mass spectrometric analysis reveals discrete chemical structures of relevant constituents.

Key words: *Munronia pinnata*, inflammation, 5-lipoxygenase, radical scavenger, microsomal prostaglandin E_2 synthase-1, mass spectrometry.

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Munronia pinnata (Wall.) Theob.: Unveiling phytochemistry and dual inhibition of 5-lipoxygenase and microsomal prostaglandin E_2 synthase (mPGES)-1

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ABSTRACT

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Conclusions: Together, potent inhibition of 5-LO and mPGES-1 activity, without concomitant antioxidant activity and cytotoxic effects, rationalizes the ethnopharmacological use of *M. pinnata* as anti-inflammatory remedy. Detailed chromatographic/mass spectrometric analysis reveals discrete chemical structures of relevant constituents.

1. Introduction

The genus *Munronia* Wight (Meliaceae) comprises 13–15 species naturally distributed in southern China, Vietnam, Myanmar, Java, Sri Lanka, India, Indonesia and the Philippines (Qi et al., 2003). *Munronia pinnata* (Wall.) Theob. (synonyms – *Munronia pumila* Wight, *Melia pumila* Moon), locally known as "Binkohomba", is a small herb widely used in Ayurvedic and folk medicine in Sri Lanka for hundreds of years (Hapuarachchi et al., 2011a). This plant is a rare species and grows in intermediate and wet zone forests and on rocky places in Sri Lanka but is also distributed in Southern and Northern India, China, Vietnam, Burma, Thailand and Timor (Dassanayake et al., 1995). The whole plant is used for commercial purposes (Hapuarachchi et al., 2011a) and is considered to be one of the most expensive plant materials (US$ 50–110/kg) used in traditional medicine in Sri Lanka (Dharmadasa et al., 2011). Nowadays much attention is drawn on the development of *ex situ* conservation methods *via in vitro* propagation techniques in order to establish commercial cultivations of this plant (Senarath et al., 2007).

In Sri Lankan folk medicine, the plant is a major ingredient of decoctions and powders used for the treatment of fever, dysentery, skin diseases, purification of blood upon snake bites and malaria

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(Jayaweera, 1982), and to prevent hiccups, vomiting and sore throats (Arambewela and Wijesinghe, 2006). According to the pharmacopoeia, it exhibits wound purifying, anthelmintic, carminative and laxative properties, it improves digestive power, reduces dermatitis, promotes lactation, destroys worms and interestingly, it is also used for the treatment of polyuria, cough and edema (Arambewela and Wijesinghe, 2006; Department of Ayurveda, 1979).

Despite its therapeutic importance, the bioactivities of *M. pinnata* are hardly explored in order to rationalize the reported ethnopharmacological use. The whole plant extract exhibits marked cytotoxicity and potent anti-malarial activity as claimed by Sri Lankan traditional practitioners (Dharmadasa et al., 2012). A pilot experimental study with aqueous extracts in healthy Wistar rats revealed statistically significant oral hypoglycemic effects (Hapuarachchi et al., 2011a,b). No acute or chronic toxic effects of water and ethanol extracts of natural plant and callus cultures of *M. pinnata* were observed in healthy rats (Hapuarachchi et al, 2013). Although the plant is extensively used to alleviate the pathological conditions caused by inflammation, pharmacological investigations on its anti-inflammatory properties are rare. A decoction of *M. pinnata* (Hapuarachchi et al., 2012) revealed anti-inflammatory effectiveness in the carrageen-induced paw edema. However, neither the anti-inflammatory principle of the plant extract nor the molecular mechanisms were identified.

Prostaglandins (PG) and leukotrienes (LTs) are formed from arachidonic acid (AA) and act as important mediators of inflammation, allergy and pain (Funk, 2001). LTs contribute to various inflammatory and allergic reactions in the pathophysiology of asthma, allergic rhinitis, atherosclerosis, cancer, etc. (Werz and Steinhilber, 2006). 5-Lipoxygenase (5-LO) that catalyzes the first two key steps in LT biosynthesis from AA is considered as valuable drug target (Radmark et al., 2007; Pergola and Werz, 2010). Among the PGs, the PGE_2 is formed from AA under inflammatory conditions essentially by cyclooxygenase (COX)-2 coupled to microsomal PGE_2 synthase (mPGES)-1 (Samuelsson et al., 2007). Dual pharmacological intervention with both LT and PGE_2 biosynthesis proposes a strong therapeutic benefit in inflammatory diseases. In fact, plant-derived natural products have been reported to dually suppress 5-LO and mPGES-1 activity (Koeberle and Werz, 2009; Werz, 2007), which rationalizes these pro-inflammatory enzymes as functional targets for anti-inflammatory phytomedicine.

The phytochemistry of *M. pinnata* is not established yet and remains to be explored. Conventional natural product isolation methodologies involving tedious chromatographic separations are extremely time consuming, technically demanding and require large quantities of sample, and are thus not feasible for phytochemical studies of rare medicinal plants like *M. pinnata*. Therefore, the development of novel methodologies which could provide detailed structural information about phytochemical constituents directly from the crude extract or less purified fractions of the crude extracts is desirable. Due to the dramatic improvement in instrumental methods in the field of mass spectrometry over the last few years, detection and identification of chemical components without extensive purification protocols is possible. Novel hyphenated techniques providing excellent separation efficiency as well as acquisition of online complementary spectrometric data from complex crude extracts enable effective compound identification in plant extracts (Sarker and Nahar, 2012). Together with the modern analytical techniques, tandem mass spectrometry (MS/MS) fragment libraries provide a potential avenue for the study of secondary metabolites at nanomole-scale. The present study was undertaken to reveal anti-inflammatory mechanisms of *M. pinnata* and to identify related secondary metabolites with novel mass spectrometric techniques.

2. Materials and methods

2.1. Plant material

Plants were collected in Weerasuriyakanda (Gampaha district, Western Province of Sri Lanka) and Algama (Kegalle district, Sabaragamuwa Province of Sri Lanka) in 2011/2012. The plant was identified by the author (MN), a botanist, and confirmed based on the books "A Revised Handbook to the Flora of Ceylon: Volume IX – M.D. Dassanayake, F.R. Fosberg and W.D. Clayton" and "Medicinal Plants (indigenous and exotic) used in Ceylon: Volume IV – D.M.A. Jayaweera" and authenticated by comparison with the herbarium specimens at the National herbarium, Royal Botanical Garden, Peradeniya, Sri Lanka. A voucher specimen (Mun-SP-1-0606) is deposited at Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Sri Lanka.

2.2. Preparation of crude extracts

The plant materials (whole plant) were thoroughly washed, chopped into small pieces and dried in shade (30 ± 2 °C) for four days. Dried plants were powdered using an electrical grinder (Singer, model: KA-MIXEE) and the powdered material (15 g) successively extracted with 600 ml of *n*-hexane, dichloromethane, ethyl acetate (EtOAc) and methanol (Roth, Karlsruhe, Germany) at room temperature using a linear shaker for 20 minutes. Besides, 3.3 g of powdered material was extracted in 300 ml of 70% methanol–water in the presence of 0.05% acetic acid by heating for 2 hours at 60 °C. Evaporation of each solvent under reduced pressure (BÜCHI- Rotary evaporator, *R*-114, Germany) yielded dried crude extracts which were then subjected to the bioactivity studies.

2.3. Evaluation of bioactivity

2.3.1. 5-Lipoxygenase (5-LO) activity in intact neutrophils

Human neutrophils were isolated from leukocyte concentrates obtained from the University Hospital Jena, Germany. In brief, peripheral blood was withdrawn from fasted (12 h) healthy donors that had not taken any anti-inflammatory drugs during the last 10 days by venipuncture in heparinized tubes (16 IE heparin/ml blood). The blood was centrifuged at 4000*g* for 20 min at 20 °C. Leukocyte concentrates were subjected to dextran sedimentation and centrifugation on Nycoprep cushions (PAA Laboratories, Linz, Austria). Contaminating erythrocytes of pelleted neutrophils were lysed by hypotonic lysis. Neutrophils were washed twice in ice-cold PBS and finally resuspended in PBS pH 7.4 containing 1 mg/ml glucose and 1 mM $CaCl_2$ (PGC buffer) (purity > 96–97%). The cells were preincubated for 15 min at 37 °C with test compounds or vehicle (0.1% DMSO), and incubated for 10 min at 37 °C with the Ca^{2+}-ionophore A23187 (2.5 μM) plus 20 μM AA. Then, the reaction was stopped on ice by addition of 1 ml of methanol, 30 μl 1 N HCl and 500 μl PBS, and 200 ng prostaglandin B_1 was added. The samples were subjected to solid phase extraction on C18-columns (100 mg, UCT, Bristol, PA, USA) and 5-LO products (LTB_4 and its trans-isomers, 5-H(P)ETE) were analyzed by HPLC on the basis of the internal standard PGB_1. Cysteinyl-LTs C_4, D_4 and E_4 were not detected (amounts were below detection limit), and oxidation products of LTB_4 were not determined.

2.3.2. 5-LO activity in cell-free assays (purified 5-LO)

Escherichia coli (BL21) was transformed with pT3-5-LO plasmid, and recombinant 5-LO protein was expressed at 30 °C as described (Fischer et al., 2003). Cells were lysed in 50 mM triethanolamine/HCl pH 8.0, 5 mM EDTA, soybean trypsin inhibitor (60 μg/ml),

1 mM phenylmethanesulphonyl fluoride, and lysozyme (1 mg/ml), homogenized by sonication (3 × 15 s), and centrifuged at 40,000g for 20 min at 4 °C. The 40,000g supernatant was applied to an ATP-agarose column to partially purify 5-LO as described previously (Fischer et al., 2003). Aliquots of semi-purified 5-LO were immediately diluted with ice-cold PBS containing 1 mM EDTA, and 1 mM ATP was added. Samples were preincubated with the test compounds or vehicle (0.1% DMSO) as indicated. After 10 min at 4 °C, samples were pre-warmed for 30 s at 37 °C, and 2 mM $CaCl_2$ plus 20 μM AA was added to start 5-LO product formation. The reaction was stopped after 10 min at 37 °C by addition of 1 ml ice-cold methanol, and the formed metabolites were analyzed by RP-HPLC as described (Fischer et al., 2003). 5-LO products include the all-trans isomers of LTB_4 as well as 5-HPETE and its corresponding alcohol 5-HETE.

2.3.3. Determination of mPGES-1 activity

Preparation of A549 cells and determination of the activity of mPGES-1 was performed as described previously (Koeberle et al., 2008). In brief, IL-1β-treated A549 cells overexpressing mPGES-1 were sonicated and the microsomal fraction was prepared by differential centrifugation at 10,000g for 10 min and at 174,000g. The resuspended microsomal membranes were preincubated with the test compounds or vehicle (DMSO). After 15 min, PGE_2 formation was initiated by addition of PGH_2 (final concentration, 20 μM). After 1 min at 4 °C, the reaction was terminated, and PGE_2 was separated by solid-phase extraction (RP-18 material) and analyzed by RP-HPLC as described (Koeberle et al., 2008).

2.3.4. DPPH assay

The radical scavenger capability was assessed by measuring the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) as described (Blois, 1958). Briefly, 100 μl of extracts (20 and 100 μg/ml) were added to 100 μl of a solution of the stable free radical DPPH in ethanol (50 μM, corresponding to 5 nmol), buffered with acetate to pH 5.5, in a 96-well plate. The absorbance was recorded at 520 nm (Multiskan Spectrum Reader, Thermo Fisher Scientific Oy, Vantaa, Finland) after 30 min incubation under gentle shaking in the dark. Ascorbic acid and L-cysteine were used as reference compounds. All analyses were performed in triplicates.

2.3.5. Measurement of reactive oxygen species in neutrophils

Neutrophils (10^7/ml PG buffer) were preincubated with test compounds (or 0.1% DMSO as vehicle) for 15 min. Then, the peroxide-sensitive fluorescence dye 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, 1 μg/ml) and $CaCl_2$ (1 mM) were added 2 min prior addition of phorbol myristate acetate (PMA, 0.1 μM). The fluorescence emission at 530 nm was measured after excitation at 485 nm in a thermally controlled (37 °C) NOVOstar microplate reader (BMG Labtechnologies GmbH, Offenburg, Germany).

2.3.6. Statistical analysis

Data are expressed as mean ± S.E.M. IC_{50} values were calculated from averaged measurements at 3–5 different concentrations of the compounds by nonlinear regression using GraphPad Prism software (San Diego, CA) one site binding competition. Statistical evaluation of the data was performed by one-way ANOVA followed by a Bonferroni or Tukey–Kramer post hoc test for multiple comparisons respectively. A p value < 0.05 (*) was considered significant.

2.4. Phytochemical screening

2.4.1. Bioassay-guided fractionation

M. pinnata hexane extract (130 mg) was dissolved in dichloromethane (about 100 μl) and was absorbed into silica gel (15 mg) (silica gel 60, 0.04–0.063 mm, 230–400 mesh, Roth, Germany), the solvents were completely removed by rotary evaporation and fractionated over a silica gel (12 g) column. The sample was eluted with *n*-hexane, 3% EtOAc in *n*-hexane, 5% EtOAc in *n*-hexane, 10% EtOAc in *n*-hexane, 15% EtOAc in *n*-hexane, 25% EtOAc in *n*-hexane, 35% EtOAc in *n*-hexane, 50% EtOAc in *n*-hexane, 75% EtOAc in *n*-hexane, EtOAc and methanol, successively, yielding 11 fractions. The collected fractions were evaporated and the dry weight was measured and subjected to bioactivity assays and GC/LC–MS analysis.

2.4.2. Liquid chromatography coupled mass spectrometric (LC–MS) analysis

The *n*-hexane extract and the fractions obtained thereof were analyzed on a LTQ-Orbit rap instrument (Thermo Fisher, San Jose, CA) with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). The samples were dissolved in ethyl acetate (LC–MS grade; 1 mg/ml) and diluted to 10 μg/ml. Fifteen μl aliquots of the diluted samples were injected and separated by liquid chromatography using a Dionex–Acclaim® RSLC 120 C18 column (2.1 mm × 150 mm packed with 2.2 μm, 120 Å). Reversed phase UPLC gradient separations were performed using (A) water (LC–MS grade), with 0.1% formic acid (LC–MS grade), and (B) methanol (LC–MS grade), with 0.1% formic acid as mobile phases. The gradient program was set as 0 min – 100% A, 0.3 ml/min, 5 min – 100% A, 0.3 ml/min, 48 min – 100% D, 0.3 ml/min, 60 min – 100% D, 0.3 ml/min, 60.1 min – 100% A, 0.3 ml/min, 65.1 min – 100% A, 0.3 ml/min. All LC–MS grade solvents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

In the ESI source, the heated capillary temperature was 275 °C and the capillary voltage and tube lens voltage were set to 48 V and 95 V, respectively. The APCI source was operated at 400 °C, the heated capillary temperature was 220 °C and the corona discharge current was set to 4.5 μA. The full scan and collision-induced dissociation (CID) mass spectra were generated using 30,000 and 7500 full width at half maximum (fwhm) resolutions, respectively. The full scan mass spectra were recorded in the m/z range 100–2000. CID mass spectra were obtained at different collision energies between 1 and 55 eV. The activation time was set at 30 ms with the activation parameter $q = 0.25$. An isolation window of 1.0 mass unit was used.

2.4.3. Molecular formula identification

Following a published method (Rasche et al., 2012), the molecular formula were identified by isotope pattern and fragmentation tree analysis. Fragmentation trees annotate fragment peaks with molecular formulas and model fragmentation reactions through dependencies between fragment ions. The fragmentation tree that explains the data best is calculated by an optimization algorithm. The score of a tree takes into account mass deviation between peak masses and assigned molecular formulas, plausibility of molecular formulas, intensity of explained peaks, and whether losses are common.

2.4.4. Gas chromatography coupled mass spectrometric (GC–MS) analysis

GC–MS analysis of the *n*-hexane extract and the fractions of interest (F-6 and F-8) was carried out on a gas chromatograph HP6890 (Agilent, CA, USA) connected to a MS02 mass spectrometer from Micromass (Waters, Manchester, UK) with EI 70 eV

equipped with ZB5ms column (30 m × 0.25 mm, 0.25 µm film thickness; Phenomenex, CA, USA). Helium was used as the carrier gas at the flow rate of 1 ml/min. The injector temperature was kept at 250 °C and the oven temperature was programmed as 100 °C (2 min), 15 °C/min to 200 °C, 5 °C/min to 305 °C (20 min).

3. Results

3.1. Evaluation of 5-LO inhibition

Isolated human neutrophils that are stimulated with the Ca^{2+}-ionophore A23187 are a well-established cell-based model for investigating the suppression of 5-LO activity by small molecule inhibitors, and was thus applied as test system in the present study. A potent inhibition of 5-LO activity in neutrophils was observed for the *n*-hexane and dichloromethane (DCM) extracts of *M. pinnata* (10 and 100 µg/ml, Fig. 1A), whereas methanol and water extracts were almost ineffective and reduced 5-LO activity only by 15 and 16% at 100 µg/ml, respectively (Fig. 1A). More detailed concentration–response studies using this cell-based assay revealed an IC_{50} value of 8.7 µg/ml for the *n*-hexane extract (Fig. 1B). The synthetic reference control inhibitor zileuton (approved as anti-asthmatic drug, Israel et al., 1990) blocked 5-LO activity with IC_{50} = 0.13 µg/ml (corresponding to 0.55 µM). Note that unspecific detrimental effects of the extracts on the viability of neutrophils can be excluded based on the ability of the cells to prevent trypan blue uptake in the presence of 10 or 100 µg/ml extract (not shown).

The pharmacological intervention with 5-LO product synthesis in the cell may be caused by diverse mechanisms, others than interference with the 5-LO enzyme activity, such as inhibition of AA supply, blockade of 5-LO-activating protein (FLAP), or loss of cell viability (Werz and Steinhilber, 2005). Therefore, the evaluation of

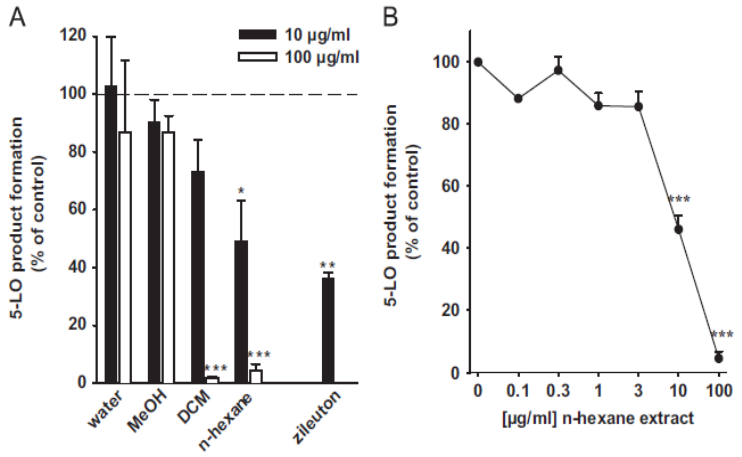

Fig. 1. Inhibition of 5-LO activity in intact neutrophils stimulated with 2.5 µM A23187 plus 20 µM AA. (A) Inhibition of 5-LO activity by various extracts of *M. pinnata*. (B) Concentration–response analysis for the *n*-hexane extract of *M. pinnata*. Neutrophils were preincubated with the extracts, zileuton (3 µM) or vehicle (0.1% DMSO) for 10 min at 37 °C prior to stimulation for another 10 min. Data are given as mean ± S.E.M., n=3–4. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* 100% control.

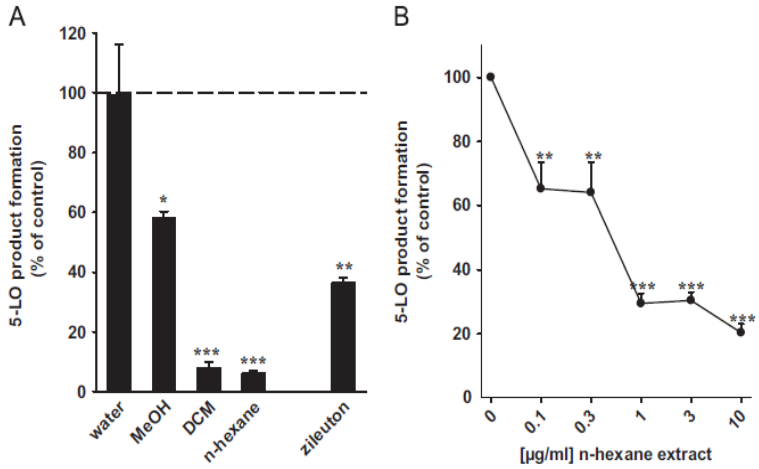

Fig. 2. Inhibition of 5-LO activity in a cell-free assay, where isolated human recombinant 5-LO was used as enzyme source and 20 µM AA as substrate. (A) Inhibition of 5-LO by various extracts (10 µg/ml) or zileuton (3 µM) of *M. pinnata*. (B) Concentration–response analysis for the *n*-hexane extract. Data are given as mean ± S.E.M., n=3–4. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* 100% control.

direct interaction of the extracts with 5-LO itself is important. In order to evaluate whether the extracts directly inhibited 5-LO activity, a cell-free assay using isolated human recombinant 5-LO as enzyme source and 20 μM AA as substrate was employed. Again, extracts (at 10 μg/ml) based on *n*-hexane or DCM as solvent efficiently blocked 5-LO activity while extracts prepared with water or methanol were much less effective (Fig. 2A). As shown in Fig. 2B, the *n*-hexane extract of *M. pinnata* caused potent and concentration-dependent inhibition of 5-LO activity with IC_{50} =0.48 μg/ml. For zileuton, the IC_{50} value was determined at 0.11 μg/ml (not shown), implying comparably marked 5-LO inhibitory potencies of the *M. pinnata* extract.

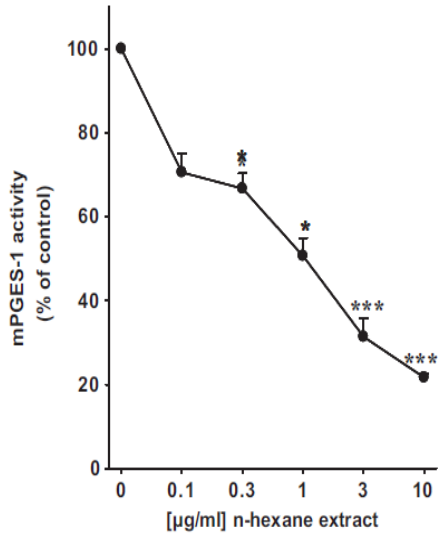

Fig. 3. Inhibition of mPGES-1 by the *n*-hexane extract of *M. pinnata*. The microsomal fraction of IL-1β-stimulated A549 cells was preincubated (10 min) with the extract and then stimulated by addition of 20 μM PGH_2. After 1 min at 4 °C, the formation of PGE_2 was assessed by RP-HPLC. Data are given as mean ± S.E.M., n=3–4. *$p < 0.05$, ***$p < 0.001$ *versus* 100% control.

3.2. Evaluation of mPGES-1 inhibition

Because the *n*-hexane extract appeared to be most interesting, we investigated the potential of this extract to interfere also with the formation of the pro-inflammatory PGE_2 produced by mPGES-1. Using the microsomal fraction of IL-1β stimulated A549 cells that strongly express mPGES-1 under these conditions, the extract potently and concentration-dependently inhibited the enzymatic transformation of PGH_2 to PGE_2, catalyzed by mPGES-1. The IC_{50} value was determined at 1.0 μg/ml (Fig. 3), which is even slightly lower than for MK886 (1.3 μg/ml = 2.4 μM), a well-recognized mPGES-1 inhibitor, used as control (not shown).

3.3. Evaluation of radical scavenging properties and suppression of ROS formation in neutrophils

Many natural products from plant origin are proposed to interfere with their molecular targets by unselective antioxidant reactions. In the case of 5-LO, they may reduce the active-site iron, decompose 5-LO-activating lipid hydroperoxides, or scavenge intermediate fatty acid radicals within LT synthesis (Werz, 2007). Therefore, radical scavenging properties of the *M. pinnata* extracts were assessed using the cell-free DPPH assay, in order to investigate whether such unselective antioxidant properties may account for 5-LO inhibition. We find that the *n*-hexane extract of *M. pinnata* up to 50 μg/ml is not able to significantly reduce radical formation, in contrast to the reference antioxidant compounds ascorbic acid or *L*-cysteine (Fig. 4A). This suggests that 5-LO inhibition by *M. pinnata* extract is not mediated by a redox-based mechanism.

In addition, the ability of the extracts (10 μg/ml) to prevent cellular ROS formation in neutrophils stimulated with the bacterial peptide fMLP was assessed. The *n*-hexane extract of *M. pinnata* did not cause significant inhibition of ROS formation, whereas DPI used as reference inhibitor completely prevented ROS generation (Fig. 4B). Of interest, extracts based on water, methanol or DCM

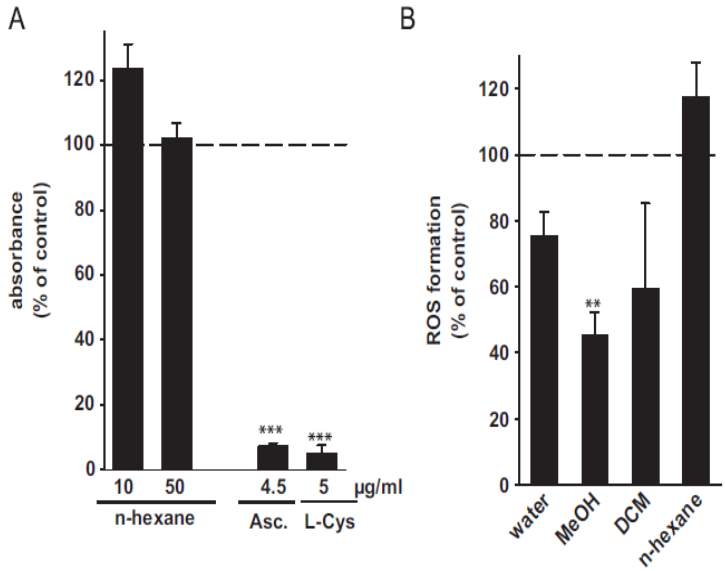

Fig. 4. (A) Radical scavenging activity of the *n*-hexane extract of *M. pinnata*. (B) Effects of the extracts of *M. pinnata* (10 μg/ml) on cellular ROS formation in neutrophils stimulated with fMLP. Neutrophils were preincubated with the extracts (or 0.1% DMSO as vehicle) for 15 min prior to stimulation with fMLP. Data are given as mean ± S.E.M., n=3–4. **$p < 0.01$, ***$p < 0.001$ *versus* 100% control.

Table 1
Inhibition of 5-LO and mPGES-1 activity in cell-free assays by fractions of the *n*-hexane extract. Data are given as mean ± S.E.M., $n=3$–4.

Fraction (no.)	Total amount of n-hexane extract (mg)	mPGES-1 residual activity (in %)		5-LO (cell-free) residual activity (in %)	
		10 μg/ml	1 μg/ml	10 μg/ml	1 μg/ml
F-1	1	n.d.	n.d.	n.d.	n.d.
F-2	4	80 ± 5	125 ± 17	60 ± 10	n.d.
F-3	1	n.d.	n.d.	n.d.	n.d.
F-4	24	n.d.	n.d.	n.d.	n.d.
F-5	8	58 ± 6	101 ± 13	57 ± 12	n.d.
F-6	22	29 ± 4	65 ± 6	29 ± 4	78 ± 8
F-7	17	15 ± 5	66 ± 6	40 ± 10	64 ± 4
F-8	1	11 ± 5	42 ± 0	30 ± 9	64 ± 5
F-9	6	49 ± 2	103 ± 1	27 ± 7	72 ± 6
F-10	6	53 ± 2	91 ± 3	64 ± 11	n.d.
F-11	9	55 ± 2	96 ± 3	50 ± 9	n.d.

were able to reduce ROS formation, with the methanol extract being most potent (54.7 ± 6.9% inhibition).

3.4. Bioassay-guided separation of the M. pinnata n-hexane extract

Early attempts of phytochemical screening of *M. pinnata* either failed (Arambewela and Wijesinghe, 2006) or provided incomplete information on an acid with 15 carbon atoms and a triterpenoid with 34 carbon atoms (Munasinghe, 2002) in a lipophilic fraction. In order to get more insights into the identity of secondary metabolites that are responsible for the potent inhibition of 5-LO and mPGES-1, the *n*-hexane extract was fractionated by liquid column chromatography using ethyl acetate, *n*-hexane and methanol. This yielded 11 fractions that were analyzed for inhibition of isolated 5-LO and mPGES-1 in the cell-free assays at 1 and 10 μg/ml, each. Out of the 11 fractions (F) of the *n*-hexane extract, F-5 (15% EtOAc in *n*-hexane), F-6 (25% EtOAc in *n*-hexane), F-7 (35% EtOAc in

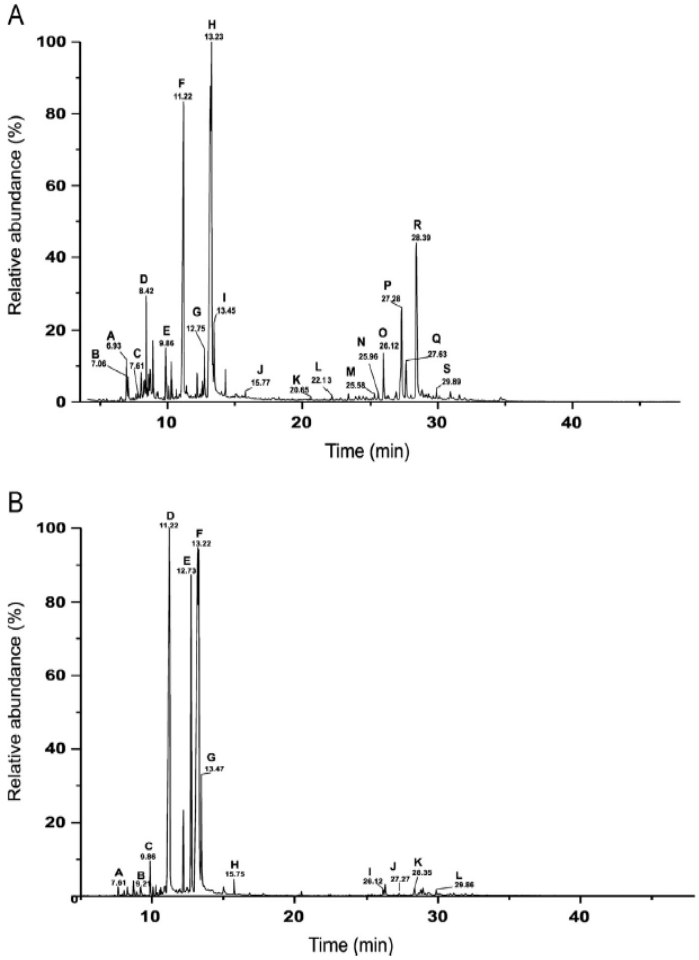

Fig. 5. (A) Total ion chromatograph of the *n*-hexane extract of *M. pinnata* and its identified compounds. **A**: β-Caryophyllene, **B**: isocaryophyllene, **C**: dodecanoic acid, **D**: caryophyllene oxide, **E**: neophytadiene, **F**: hexadecanoic acid, **G**: phytol, **H**: 9,12-octadecadienoic acid, **I**: octadecanoic acid, **J**: 4,8,12,16-tetramethylheptadecan-4-olide, **K**: heptacosane, **L**: squalene, **M**: stigmastan-3,5-diene, **N**: hentriacontane, **O**: α-tocopherol, **P**: campesterol, **Q**: stigmasterol, **R**: β-sitosterol, and **S**: stigmast-4-en-3-one. (B) Total ion chromatograph of F-6 of the *n*-hexane extract of *M. pinnata* and its identified compounds. **A**: dodecanoic acid, **B**: tetradecanoic acid, **C**: neophytadiene, **D**: hexadecanoic acid, **E**: phytol, **F**: 9,12-octadecadienoic acid, **G**: octadecanoic acid, **H**: 4,8,12,16-tetramethylheptadecan-4-olide, **I**: α-tocopherol, **J**: campesterol, **K**: β-sitosterol, and **L**: stigmast-4-en-3-one.

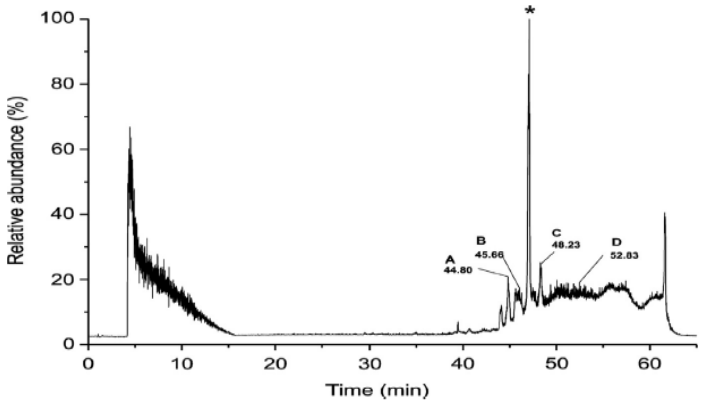

Fig. 6. Total ion chromatograph of F-6 of the *n*-hexane extract of *M. pinnata* when analyzed with the ESI source and the presumed compounds. **A**: Ganoderiol *F*, **B**: conicasterol C/theonellasterol, **C**: stigmastentriol, **D**: stigmasterol (the peak denoted as "*" is due to erucylamide, a contaminant in the LC system).

n-hexane), F-8 (50% EtOAc in *n*-hexane), F-9 (75% EtOAc in *n*-hexane) and F-11 (MeOH) at a concentration of 10 μg/ml inhibited 5-LO activity as well as mPGES-1 by >50%. Among these fractions, F-6, F-7, F-8, and F-9 were significantly active at 1 μg/ml for 5-LO whereas F-6, F-7, and F-8 inhibited mPGES-1 significantly at 1 μg/ml (Table 1). These data imply a good correlation of the fractions for dual inhibition of 5-LO and mPGES-1.

3.5. Identification of constituents of the n-hexane extract and its bioactive fractions

3.5.1. GC–MS analysis

The *n*-hexane extract and the fractions F-6 and F-8, which displayed high 5-LO and mPGES-1 inhibitory activities, were subjected to a phytochemical screening by GC–MS and UPLC–MS. The GC–MS analysis of the *n*-hexane crude extract led to the identification of 19 components, revealed by comparison of their experimental mass spectrum with those recorded in the NIST MS Search 2.0, Adams and Wiley mass spectrum libraries and also by the comparison with the respective standards. These components include ubiquitously occurring fatty acids (dodecanoic acid, hexadecanoic acid, 9,12,-octadecadienoic acid, octadecanoic acid), sesquiterpenes (*β*-caryophyllene, isocaryophyllene, caryophyllene oxide), a diterpene alcohol (phytol), an acyclic diterpene (neophytadiene), higher alkanes (heptacosane, hentriacontane), a triterpene (squalene) as well as the isoprenoid 4,8,12,16-tetramethylheptadecan-4-olide and α-tocopherol (Fig. 5A). Among the above identified compounds, 12 compounds were detected in F-6 (absolute amount 22 mg) and only 3 compounds in F-8 (absolute amount 1 mg, see below) after analysis. The total ion chromatogram (TIC) of F-6 is shown in Fig. 5B.

3.5.2. LC–MS analysis

The accurate mass measurements and the subsequent database search in METLIN suggested several compounds for the peaks denoted as compounds **A**, **B**, **C** and **D** present in F-6 (Fig. 6). Relatively poor fragmentation in the MS/MS experiments obstructed the exact compound identification, nevertheless the analysis of available CID spectra and the fragmentation trees constructed from the CID spectra eliminated many of the possibilities for the given *m*/*z* value and proposed the best fitting compound.

Compound **A**, with a *m*/*z* value of 477.33337, fits the molecular formula of $C_{30}H_{46}O_3Na$ with a mass accuracy of −1.145 ppm. The APCI-MS measurements (not shown) also supported this molecular formula where the protonated adduct ($C_{30}H_{47}O_3$) with a *m*/*z* value of 455.35220 was observed in the spectra with a mass accuracy of −0.703 ppm. The comprehensive analysis of the fragmentation pattern (Figs. S1 and S2) suggested the most possible structure for compound **A** as ganoderiol *F*.

The accurate mass measurements of compound **B** from the Orbitrap instrument suggested the molecular composition of $C_{30}H_{50}O_3Na$ (*m*/*z* 481.36490) with a mass accuracy of −0.658 ppm. The database search suggested several compounds that fit with this molecular formula and mass accuracy, however, the analysis of CID spectra excluded many possibilities and proposed compound **B** to be most likely the triterpenoids, conicasterol C or theonellasterol E according to its characteristic fragmentation pattern (Fig. S3).

In addition, compound **C** represents the molecular composition of $C_{29}H_{50}O_3Na$ (*m*/*z* 469.36456) with a mass accuracy of −0.802 ppm and compound **D** as $C_{29}H_{49}O$ (*m*/*z* 413.37775) with a mass accuracy of −0.043 ppm. Hence, these two compounds are proposed as stigmastentriol and stigmasterol, respectively.

In F8, three compounds were found, two of them (*i.e.*, ganoderiol F with *m*/*z* 477.33337 and conicasterol C or theonellasterol E with *m*/*z* 481.36490) are the same as in F-6 and the third compound with *m*/*z* 479.34921 represents the molecular formula of $C_{30}H_{48}O_3Na$ with a mass accuracy of −0.744 ppm. However, it could not be identified due to the poor fragmentation.

4. Discussion

M. pinnata belongs to the most important medicinal plants in Sri Lanka. However, the knowledge on its pharmacological features, phytochemistry, and its bioactive constituents is insufficient to explain the therapeutic use in the treatment of inflammation-related disorders. Therefore, the present study was conducted in order to rationalize and validate its traditional use as anti-inflammatory remedy by analysis of its ability to interfere with typical pro-inflammatory drug targets (*i.e.*, 5-LO and mPGES-1) and by chemical profiling of the bioactive fractions. In fact, the *n*-hexane extract of *M. pinnata* caused direct and potent inhibition of human mPGES-1 and 5-LO and suppressed the biosynthesis of 5-LO products also in intact human neutrophils. The structural characterization of potential bioactive secondary metabolites was achieved by rapid and convenient mass spectrometric approaches.

In our study, focus was placed on mPGES-1 and 5-LO as potential targets, and interference with these enzymes might help to explain the anti-inflammatory properties of *M. pinnata*. 5-LO

plays a key role in the biosynthesis of the pro-inflammatory LTs (Radmark et al., 2007) and has therefore been intensively explored as drug target for the intervention with asthma, allergic rhinitis, various autoimmune diseases, cardiovascular disease, cancers, and many other inflammatory disorders (Peters-Golden and Henderson, 2007; Werz and Steinhilber, 2006). According to the molecular mode of action, direct 5-LO inhibitors (of synthetic or natural origin) are categorized as (I) redox-type inhibitors that interfere with the redox cycle of the 5-LO active site iron, (II) iron ligand-type inhibitors that chelate the active site iron, and (III) nonredox-type inhibitors that compete with AA as substrate and/or activating fatty acid hydroperoxides or act on 5-LO by so far unrecognized mechanisms (Ford-Hutchinson et al., 1994; Werz and Steinhilber, 2005). Several hundred plants and their extracts and/or specific secondary metabolites have been investigated as inhibitors of the biosynthesis of 5-LO products (Yoshimoto et al., 1983; Laughton et al., 1991; Werz, 2007; Schneider and Bucar, 2005). In fact, the plant kingdom appeared as potential source for 5-LO inhibitors and the interference with 5-LO activity is considered as basis for the anti-inflammatory features of the respective plants (and medical preparations thereof) in folk medicine (Schneider and Bucar, 2005). However, the 5-LO inhibitory potencies of the investigated extracts often turned out to be comparably low and relatively high IC_{50} values in the rough range of 15–50 μg/ml for 5-LO inhibition were observed for lipophilic extracts of various medicinal plants (Schneider and Bucar, 2005). Thus, the IC_{50} of 8.7 μg/ml in neutrophils and of 0.48 μg/ml in the cell-free 5-LO activity assay obtained for the *n*-hexane extract of *M. pinnata* is remarkable and suggests a high pharmacological potential for intervention with 5-LO-related disorders.

Since most of the plants are rich in anti-oxidants such as polyphenols, flavonoids and coumarins, the iron-cheating and antioxidant features of these compounds are often responsible for uncoupling of the 5-LO catalytic cycle and eventually for 5-LO inhibition. Interestingly, the 5-LO inhibitory *M. pinnata* extract neither exhibits significant radical scavenging nor antioxidant activities in cell-free (DPPH assay) or cell-based (ROS generation in neutrophils) test systems. This suggests that the *n*-hexane extract of *M. pinnata* may contain nonredox-related principles that interact with 5-LO. It was observed that the potency of the *n*-hexane extract was more pronounced for inhibition of isolated 5-LO, compared to cellular 5-LO. A possible explanation could be that the active constituents fail to reach equivalent concentrations inside the cell (due to poor permeation) or cellular factors may impair 5-LO inhibition by competition, unspecific protein binding or degradation of the constituents.

Previous studies showed that plant derived 5-LO inhibitors such as hyperforin, myrtucommulone, garcinol, arzanol, curcumin, boswellic acids and embelin also inhibit the activity of mPGES-1 (Koeberle and Werz, 2009; Bauer et al., 2011; Schaible et al., 2013), and such dual suppression of two major pro-inflammatory pathways might be beneficial for effective and safe therapy. PGE_2 is considered as major mediator of inflammation and pain, and non-steroidal anti-inflammatory drugs are assumed to confer their anti-inflammatory effect essentially *via* suppression of PGE_2 biosynthesis (Funk, 2001). In fact, the *n*-hexane extract of *M. pinnata* and also the fractions F-6 to F-9 effectively repressed the activity of mPGES-1 with $IC_{50} = 1$ μg/ml. To the best of our knowledge, no other medicinal plant-derived extract has been reported thus far with such high potency against mPGES-1, and also the well-recognized synthetic mPGES-1 inhibitor MK886 ($IC_{50} = 1.3$ μg/ml) was not superior. Note that a strong correlation regarding the potencies to inhibit 5-LO and to inhibit mPGES-1 for the single fractions was evident, suggesting that both targets are affected by the same ingredient(s). Because mPGES-1 is regarded as a valuable drug target for the treatment of various common disorders including pain, inflammation, fever, and cancer (Samuelsson et al., 2007), the potent suppression of mPGES-1 activity by *M. pinnata* is encouraging and supports its medical and therapeutic use. Moreover, the low IC_{50} values of 0.48–8.7 μg/ml for the *n*-hexane extract against 5-LO and mPGES-1 would roughly correspond to an estimated dose of 36–650 mg per 75 kg body weight (assuming 100% bioavailability and equal distribution) and is thus clearly in the range of the dosage of well-recognized anti-inflammatory phytomedicine used in Western countries such as extracts of nettle, willow bark, or devil's claw that are applied at about 145–600 mg/single dose. Therefore, the high efficiency of *M. pinnata* against 5-LO and mPGES-1 *in vitro* might be of pharmacological relevance and provides a rationale for its use as anti-inflammatory remedy in folk medicine.

The chemical profiling of *M. pinnata* has been hindered for many years mainly due to the dearth of plant materials for large scale extraction and isolation procedures. However, our approach for compound identification was devoid of any extensive purification, thus, required only a few grams of plant materials. Yet, the modern hyphenated techniques enabled us to unveil the phytochemistry of this medicinal plant for the first time. An UPLC system coupled to the Orbitrap instrument and GC coupled to MS were employed as the sole techniques in this study for the characterization of secondary metabolites. While the UPLC provided excellent separation efficiency, the Oribtrap mass accuracy made the determination of molecular composition of precursor and fragment ions straightforward. In addition to the MS database search for the molecular formula of the parent mass, the tandem mass spectra were further analyzed by computer assisted algorithms to yield hypothetical fragmentation trees, which provided better structural insight into the possible compounds.

Among the identified constituents in the *n*-hexane extract by GC–MS, *β*-caryophyllene and caryophyllene oxide might be of interest. Anti-inflammatory activity of *β*-caryophyllene was revealed in models of acute (carrageenan-induced) inflammation (Gertsch et al., 2008) while caryophyllene oxide exhibited significant cytotoxicity against the human cancer cell lines HepG2, AGS, HeLa, SNU-1, and SNU-16 in the MTT assay (Jun et al., 2011). Since the above-mentioned compounds were only sparsely present in the most active fractions of the *n*-hexane extract (F-6/F-8), we conclude that those are not exclusively responsible for the observed bioactivity. However, the presence of α-tocopherol in the F-6 could be correlated to the 5-LO inhibition as it has displayed a potent inhibition by selective and tight binding to 5-LO (Reddanna et al., 1985).

The LC–MS analysis of F-6 and of F-8 of the *n*-hexane extract suggests the presence of some interesting compounds, however, the available tandem mass spectral data which resulted from poor fragmentation of precursor ions, are insufficient for a conclusion. Nevertheless, we could putatively assign the structures after considering the accurate mass, mass accuracy and database search. Particularly, the tentative identification of ganoderiol F is of interest as this triterpenoid was reported to exhibit strong anti-HIV-1 protease activity (El-Mekkawy et al., 1998) as well as *in vivo* antitumor effects (Gao et al., 2006). Further optimization of LC–MS/MS conditions in planned follow-up studies may permit confirmation of the identified structures. Furthermore, the LC–MS analysis has indicated the existence of several unknown compounds that are not in any database which might have also contributed to the bioactivity. Therefore, the planned expansion of this study towards the identification of unknown compounds in the active fraction with the use of fragmentation tree alignments (Rasche et al., 2012) will provide better insights into the chemical profile of the plant. Thereafter, further experiments with synthesized compounds will be worthwhile for a better understanding of their bioactivities, in particular with respect to the inhibition of 5-LO and mPGES-1.

5. Conclusion

The potent inhibition of the activity of the drug targets 5-LO and mPGES-1 in well-established biological test systems provides scientific evidences for *M. pinnata*'s traditional usage in Sri Lanka for the treatment of various inflammatory conditions. It has been revealed that the constituents in the *n*-hexane extract of *M. pinnata* exhibit potent interference with 5-LO in a nonredox-dependent manner rather than *via* an unselective antioxidant mechanism. The phytochemistry of *M. pinnata* was revealed for the first time where phytosterols, fatty acids, sesquiterpenes and several other types of secondary metabolites with known anti-inflammatory properties were identified by mass spectrometric techniques. We believe that the solid platform laid by our study will be indispensable for further phytochemical and bioactivity research on this popular and valuable medicinal plant in the future. Our data may stimulate for more detailed preclinical analysis of the pharmacological properties of *M. pinnata* that may further support its therapeutic potential in the treatment of inflammatory disorders.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2013.11.052.

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Supplementary -1

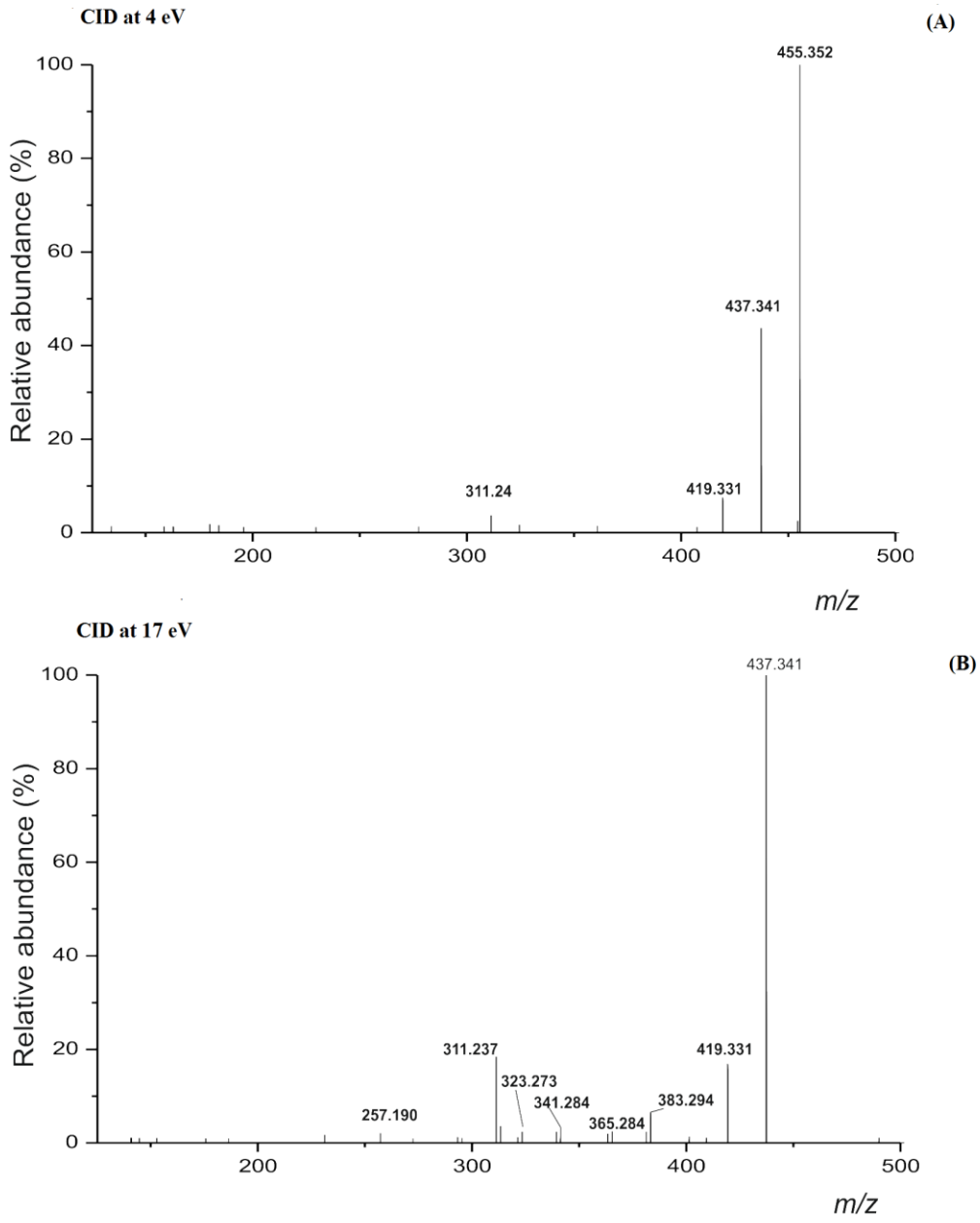

Fig. S-1: Collision induced dissociation spectra of putatively identified ganoderiol F at **(A)** 4 eV and **(B)** 17 eV

Supplementary -2

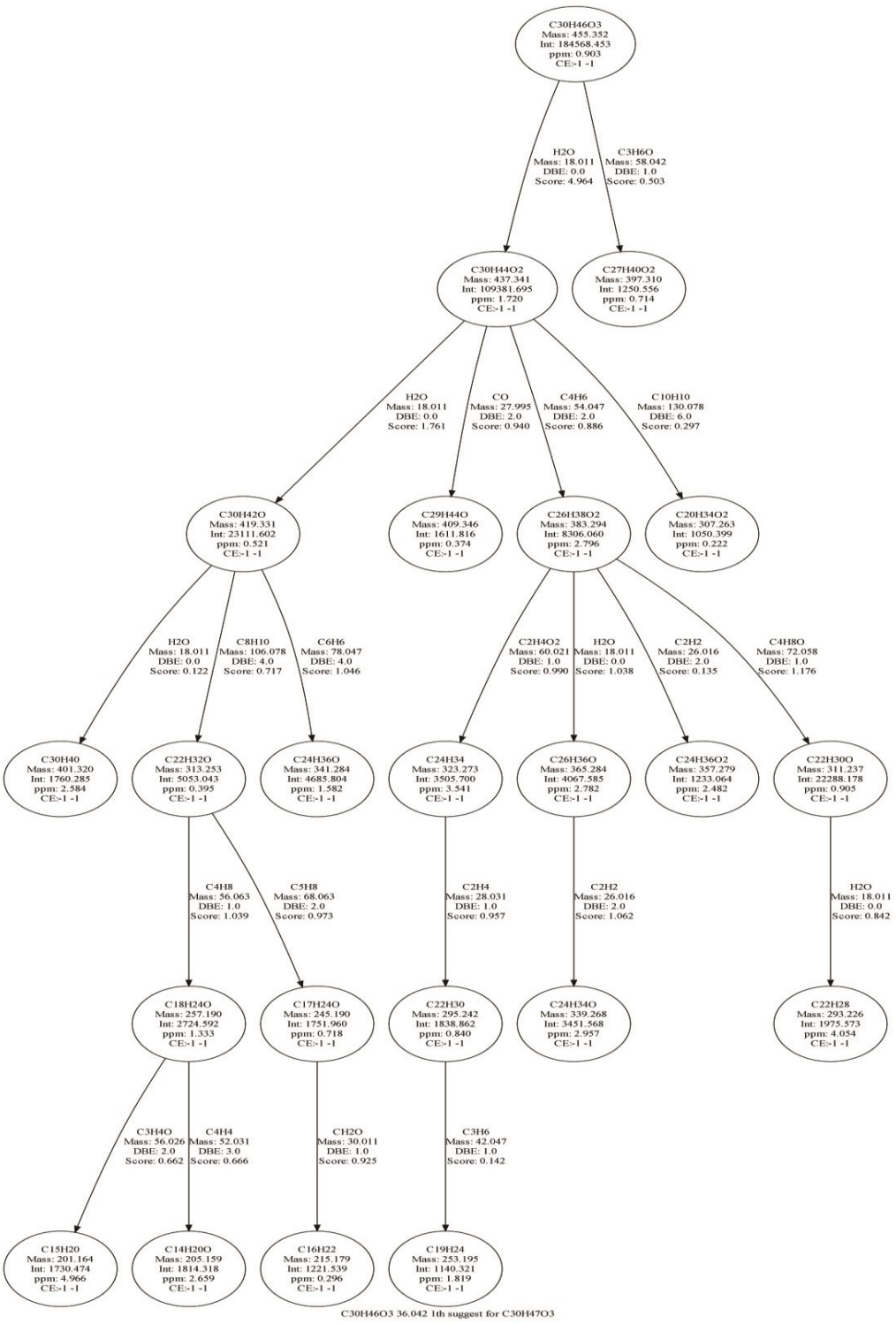

Fig. S-2: Hypothetical fragmentation tree for putatively identified ganoderiol F (protonated adduct)

Supplementary -3

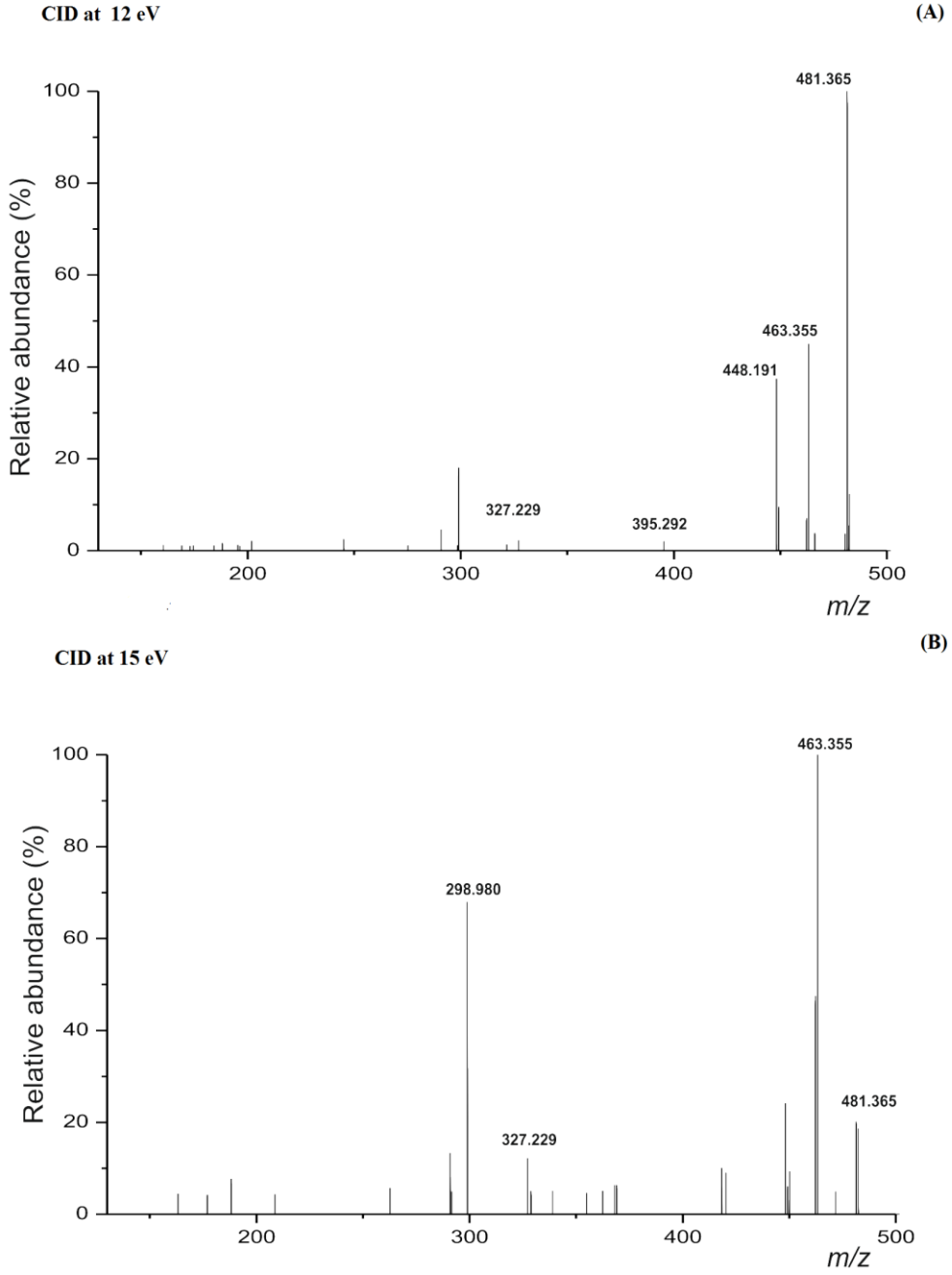

Fig. S-2: Collision induced dissociation spectra of putatively identified triterpenoid at **(A)** 12 eV and **(B)** 15 eV

Chapter 5

Manuscript-IV

Identification of Female Specific Fatty acid Derivatives in *Drosophila melanogaster* Surface Lipid Extracts

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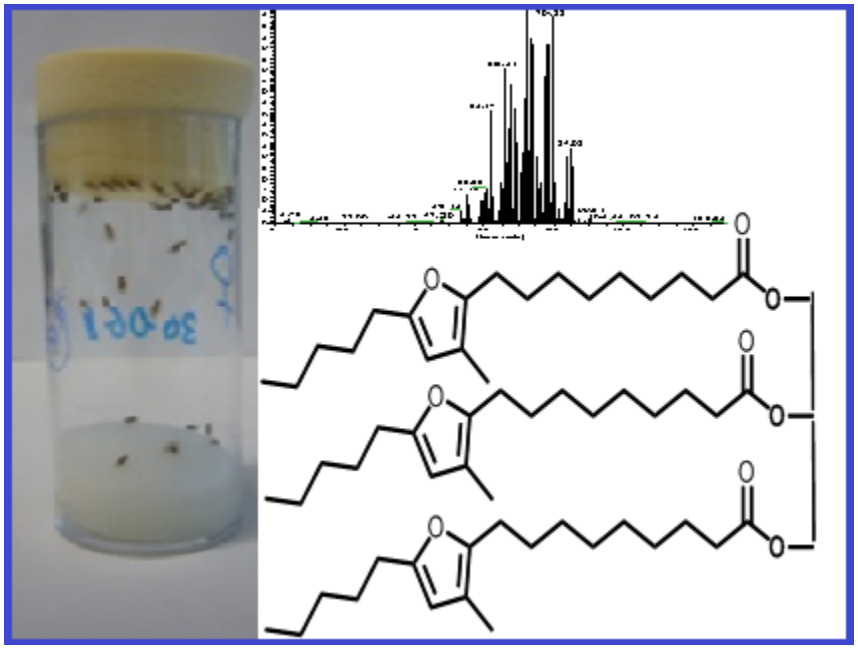
In preparation for the submission to *ChemBioChem*



Abstract

Investigations carried out so far on sex dependent differences in composition of cuticular lipids in *Drosophila melanogaster* have been exclusively focused on cuticular hydrocarbons. As a result, the understanding on non-hydocarbon components particularly fatty acid and fatty acid derivatives in the surface lipid extracts of male and female flies is rather primitive. Therefore, the identification of female specific 9-(3-methyl-5-pentylfuran-2-yl)nonanoic acid and its TAG by UHPLC-APCI-MS and GC-MS methods directs the field of insect cuticular chemistry in to new dimensions. Our finding not only contradicts the decades-old concept of the absence of qualitative differences between cuticular fatty acid profiles in male and female flies but also highlights the necessity of a detailed study on the biosynthesis and physiological functions of this female specific fatty acid.

Key words : cuticle, fatty acid derivatives, *Drosophila melanogaster*, 9-(3-methyl-5-pentylfuran-2-yl)nonanoic acid

1. Introduction

The knowledge on surface chemistry of insects is crucial for a better understanding of the physiological functions performed by the insect cuticle. Particularly the cuticular chemistry of insects has drawn much attention with the recognition of its involvement in various types of chemical communications (Howard, 1993). The advent of analytical methods enabled the determination of structures and the composition of the cuticular lipids in many insect species. Hydrocarbons were identified as the major component while esters, free alcohols, fatty acids, ketones, etc. were also detected in minor quantities in many species.

After the observation of sex dependent differences in surface lipid composition in several Diptera species, the investigations of surface lipids in *Drosophila melanogaster* were initiated few decades ago. The preliminary evaluation of *D. melanogaster* surface lipids indicated the presence of hydrocarbons; alkanes, alkenes and alkadienes in considerable amount. The study was then extended towards the determination of quantitative and qualitative differences in cuticular lipids in males and female flies (Jackson, et al., 1981) which revealed quantitative differences in hydrocarbons, acylglycerol, and free fatty acids in hexane extracts of both sexes.

Since the commencement of studies on surface lipids of *D. melanogaster*, the hydrocarbon components have undergone intensive investigations. This led to the identification of several compounds displaying marked sexual dimorphism hence performing an important role as species-specific signals (pheromones). For example, CHs with two double bonds (often 7,11-dienes) are produced only in females and stimulate male courtship while monoenes (such as 7-Tricosene) are mostly found in males (Jallon, 1984; Antony and Jallon, 1982; Foley, et al., 2007). However, the non-hydrocarbon components of surface lipids in *D. melanogaster* have not been extensively studied so far. A previous study on non-hydrocarbon lipid components revealed the presence of myristic acid, palmitic acid, palmitoleic acid, oleic acid, and linoleic acid as major fatty acids from both the acylglycerol and free fatty acid

fractions of both sexes (Jackson, et al., 1981). Despite the quantitative differences, any qualitative differences between the fatty acid compositions of either free fatty acids or acylglycerol fatty acids from male and female flies have not been observed in this study. Interestingly, any evidences to disprove this observation have not been reported yet neither.

Nowadays, mass spectrometry has turned a new era in the analysis of lipids and several mass spectrometry based techniques are widely employed in the analysis of insect surface lipids. GC-MS is a well-established tool and plays a predominant role in the detection and structure elucidation of sufficiently volatile nonpolar cuticular hydrocarbons. However, the high probability of missing larger and more polar cuticular compounds by this method appears as a major drawback in such an analysis. Thus the conventional approach involves hydrolysis of lipids followed by derivatization in to fatty acid methyl esters (FAME) and subsequent GC-MS analysis of released fatty acid derivatives. The analysis of FAMEs by GC-MS does not provide complete information about the original lipid molecule, particularly, the arrangement of constituent FAs to the glycerol back-bone. As a result, there was a demand for the development of novel approaches that facilitate the detection and identification of more polar and high molecular weight cutitular components.

With the emergence of HPLC/APCI-MS, the analysis of complex lipid mixtures has redeem a momentum as it allows for a direct analysis of triacylglyerols of higher molecular weight (Kofroňová, et al., 2009; Cvačka, et al., 2006). Depending on the mass analyzer used, high accuracy in the determination of the molecular mass can be achieved where as extrem sensitivity in mass spectrometric approaches enable the identification of even minor components in a crude lipid extract without extensive purifications.

In this study, we are attempting to investigate the presence of sex dependent fatty acids and fatty acid derivatives in the surface lipids extracts of *D. melanogaster* by

means of novel mass spectrometric approaches. Thereby, we hope to rectify the validity of the decades' old concept of the absence of qualitative differences in fatty acid profiles of male and female flies.

2. Experimental

2.1 Rearing flies

Each mature pupae of *D. melanogaster* (Canton S) was reared on corn meal sugar agar diet in an individual Eppendorf tube for 3 days at 25 °C. Thereafter, the flies of each sex were sorted out and transferred into separate vials containing 2 mL of 2% sucrose and incubated at 25 °C for 2 days. The flies were then freezed at -20 °C and the extraction of surface lipids was carried out as mentioned below.

2.2 Extraction

Two thousand and five hundred flies of each sex were superficially washed with dichloromethane : methanol (2:1) (7.5 mL) mixture. Each extract was concentrated to nearly dryness with the use of rotary evaporator (R-114, BÜCHI, Switzerland). 100 µL of the crude extract were taken out and stored at -20 °C for future references. The remaining portion of the extract was dissolved in methanol (about 100 µL) and was adsorbed into silica gel (about 100 mg) and completely evaporated the solvents. This was loaded into the column packed with silica gel (1 g) and eluted with hexane, 2% EtOAc in hexane, 5% EtOAc in hexane, 8% EtOAc in hexane, 10% EtOAc in hexane, 12% EtOAc in hexane, 15% EtOAc in hexane, 18% EtOAc in hexane, 20% EtOAc in hexane, 25% EtOAc in hexane, 30% EtOAc and methanol successively. These 12 fractions from male and female flies were subjected to spectrometric analysis.

2.3 Ultra-High Performance Liquid Chromatography-Atmospheric Pressure Chemical Ionization Mass Spectrometry (UHPLC-APCI-MS)

Non-aqueous reversed phase UHPLC separation was carried out by Dionex –Acclaim ® RSLC 120 C18 column (2.1 × 150 mm packed with 2.2 µm, 120 Å) using acetonitrile (A) and propan-2-ol (B) as mobile phases. The gradient program was set as: 0 min - 100% A 0.5 mL/min, 58 min - 30% A, 70% B, 0.5 mL/min, 70 min - 10% A, 90% B, 0.3 mL/min, 100 min - 100% A, 0.5 mL/min.

The APCI source was operated at 400 °C, the heated capillary temperature was 220 °C and the corona discharge current was set to 4.5 µA. the full scan mass spectra were recorded in the *m/z* range 150 -1400. The full scan and collision-induced dissociation (CID) mass spectra were generated using 30 000 and 7500 full width at half maximum (fwhm) resolutions respectively.

2.4 LC-MS fractionation

The above-mentioned solvent gradient was used for the collection of TAG of interest. The sub-fraction eluted at 20-24 min in the LC run fraction was collected, concentrated and half of it was trans-methanolyzed and converted into a methyl ester of a fatty acid (FAME) while the rest is subjected to different chemical reactions.

2.5 Trans-methanolysis and GC-MS analysis of the TAG

The collected fraction was evaporated and dissolved in ACN (about 1 mL) into which MSTFA (N-methyl-N-trimethylsilyl trifluoroacetamide) (20 µL) was added. The reaction mixture was heated at 65 °C for 1 hour. Thereafter, the solvents were completely evaporated. In to the residue, 0.05 M KOH in MeOH (50 µL) was added and incubated at 4 °C for 20 min. Thereafter, 1M KH_2PO_4 + $KHPO_4$ buffer (pH 5-6) (75 µL) and hexane (500 µL) was added and vortexed for 5 min. Hexane layer was taken out and any water droplets were removed by addition of Na_2SO_4. Thereafter, the sample was methylated with CH_2N_2 and injected into GC-MS.

The GC-MS measurements were executed on a gas chromatograph HP6890 (Agilent, CA, USA) connected to a MS02 mass spectrometer from Micromass (Waters, UK) with EI 70 eV using ZB5ms column (30 m × 0.25mm, 0.25-µm film thickness; Phenomenex, CA, USA). The carrier gas was helium at the flow rate of 1mL/min. The injector temperature was kept at 220 °C and the temperature program was set as 40 °C (2 min), 15°C / min to 300 °C (3 min)

2.6 Hydrogenation

Small amount of 10% Pd/C in acetone which was pre-washed with ethyl acetate was mixed with TAG sample (50 µL). The mixture was treated with H_2 for about 2 hours while stirring. Thereafter, the reaction mixture was concentrated and analyzed by LC-APCI-MS.

2.7 Oxime formation

Pentaflurobenzyl hydroxylamine hydrochloride in CH_2Cl_2 (10 µL) and one bead of molecular sieve (3A) was added to TAG sample (50 µL) and shaken for about 2 hours. Thereafter, the mixture was evaporated and finally dissolved in ACN: propan-2-ol (1:1) (50 µL) and analyzed by LC-APCI-MS. Following the same procedure, fatty acid methyl ester sample (50 µL) was treated with pentaflurobenzyl hydroxylamine hydrochloride in CH_2Cl_2 (10 µL) and the resulting mixture was analyzed by GC-MS.

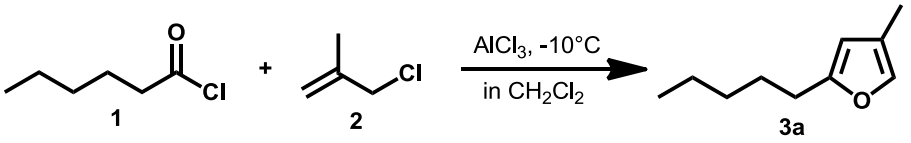
2.8 Acetylation

TAG sample (50 µL) was reacted with 10 µL of acetic anhydride and 10 µL of lutidin in CH_2Cl_2. The reaction mixture was kept for about 2 hours for the completion of the reaction. Thereafter it was evaporated and finally dissolved in 50 µL of ACN: propan-2-ol (1:1) and analyzed by LC-APCI-MS.

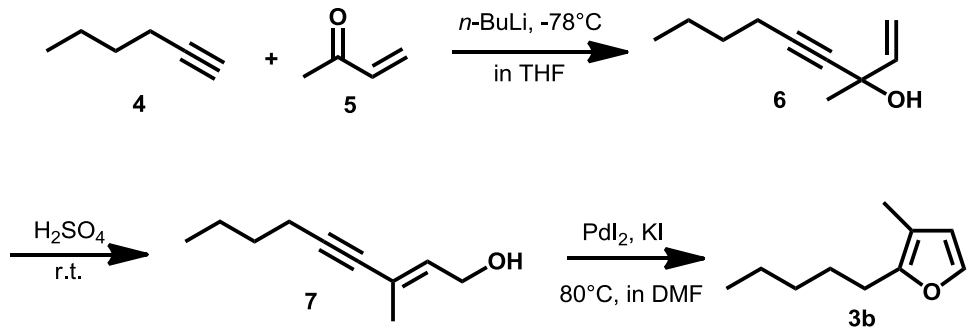
2.9 DMDS derivatization

Using an argon stream, any air present in the TAG sample/ FAME sample was removed and 5% I_2 in diethyl ether (1 drop) followed by DMDS (1 drop) was added to it. Then the reaction mixture was concentrated and kept in dark. The reaction was quenched by the addition of 10% $Na_2S_2O_3$ in water after 1 hour. The experiment was repeated following the same procedure, however the reaction was quenched after 6 hours. Then the organic layer was taken out, concentrated and analyzed by GC-MS.

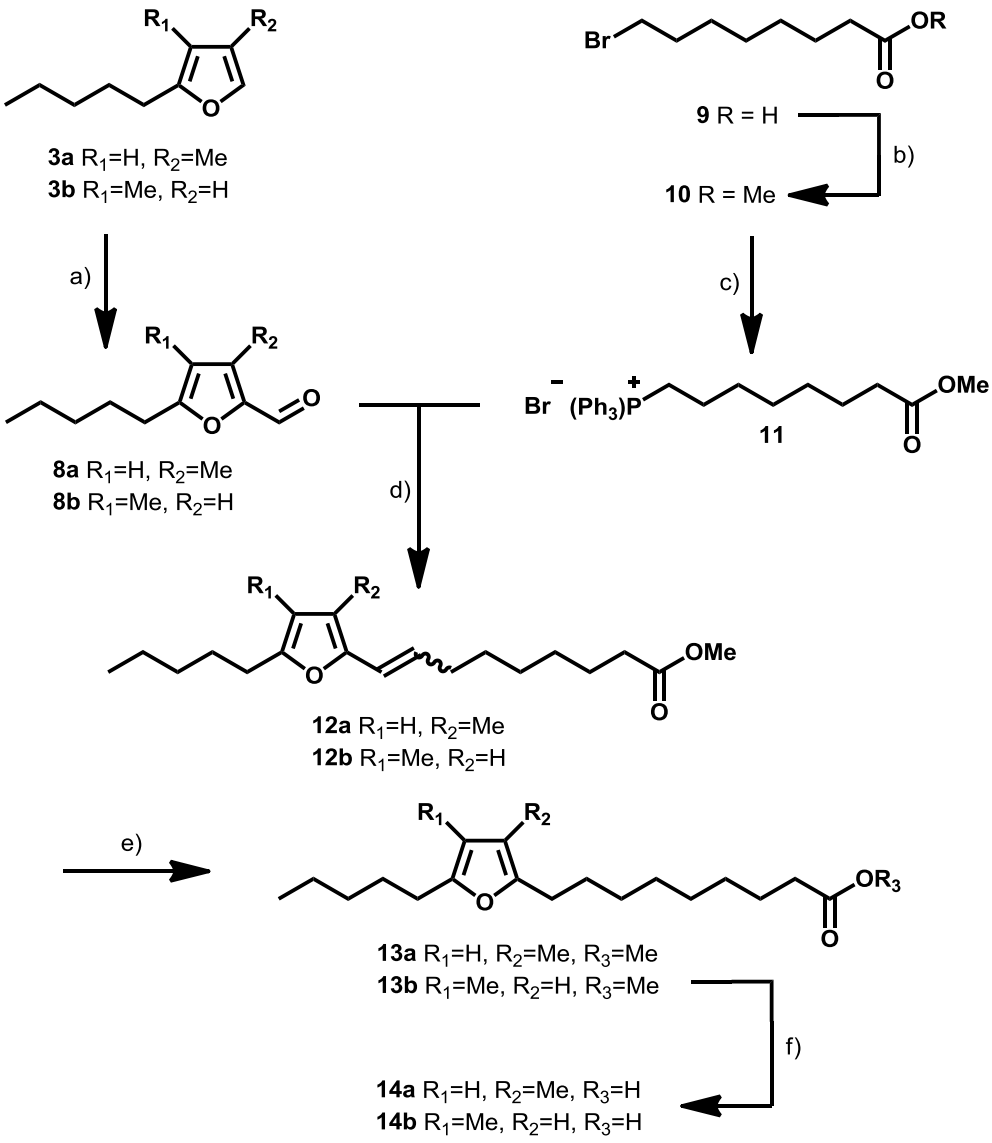
2.10 Synthesis of Fatty Acids



Scheme1: Synthesis of 4-Methyl-2-pentylfuran (**3a**)



Scheme2: Synthesis of 3-Methyl-2-pentylfuran (**3b**)



a) $POCl_3$, DMF, 0°C to r.t. b) 10% H_2SO_4, c) Ph_3P, reflux, in Xylene, d) NaOMe, 0°C to r.t., in DMF, e)H_2, cat. Pd/C, in MeOH, f) 1M NaOH, 60°C, in MeOH/THF

Scheme 3: Synthesis of 9-(3-Methyl-5-pentyl-2-furyl)-nonanoic acid and 9-(4-Methyl-5-pentyl-2-furyl)-nonanoic Acid

All chemicals were purchased from Sigma-Aldrich. NMR-spectra were obtained using a Bruker Avance DRX 500 NMR Spectrometer. MS-data were recorded on MS02 mass spectrometer from Micromass (Waters, UK).

2.10.1 Synthesis of 9-(3-Methyl-5-pentyl-2-furyl)-nonanoic Acid (14a)

9-(3-Methyl-5-pentyl-2-furyl)-nonanoic acid was synthesized from 4-methyl-2-pentylfuran (**3a**) and methyl-6-bromooctanoate (**10**) in 4 steps using the protocol of Tsukasa (1993). All spectral data were in accordance with the data in the literature.

2.10.1.a Synthesis of 4-Methyl-2-pentylfuran (3a)

25 mL (0.18 mmol) of hexanoyl chloride (**1**) and 17 mL (0.17 mmol) 3-chloro-2-methyl-1-propene (**2**) were added dropwise to a suspension of 24 g (0.18 mmol) $AlCl_3$ in 35 mL dichloromethane while maintaining the temperature at -10 to -15 °C. The mixture was stirred at the same temperature for 30 min before it was poured on crushed ice. After the separation of the phases, the aqueous layer was extracted with ethylacetate (3 × 100 mL). The combined organic phase was washed with water, sat. Na_2CO_3-solution and brine, before being dried with Na_2SO_4 and concentrated *in vacuo*. The residue was distilled (bp. 75-76 °C at 33 mbar) to give 21 g (81% yield) of 4-methyl-2-pentylfuran (**3a**) as colorless oil.

Spectral data were in accordance with those found in literature.

2.10.1.b Synthesis of Methyl-8-bromooctanoate (10)

Methyl-8-bromooctanoate (**10**) was synthesized from 8-bromooctanoic acid (**9**) using the protocol of Savariar, et al., (2006). All spectral data were in accordance with the data in the literature.

2.10.2 Synthesis of 9-(4-Methyl-5-pentyl-2-furyl)-nonanoic Acid (14 b)

2.10.2.a Synthesis of 3-Methylnon-1-en-4-yn-3-ol (6)

Hexyne (**4**) (12.6 mL 110 mmol) was dissolved in THF (200 mL) under an atmosphere of argon and cooled to -78 °C. A 1.6 M solution of *n*-BuLi in hexane (68.75 mL, 110 mmol) was added dropwise over a period of 15 min. After stirring at -78 °C for 10 min, 8.1 mL (100 mmol) of 3-buten-2-one (**5**) were added dropwise over a period of 5 min. The solution was stirred at the same temperature for further 20 min, before being allowed to warm to room temperature. The reaction was then quenched

with 100 mL water. After the separation of the phases, the aqueous layer was extracted with diethylether (3 × 50 mL). The combined organic phase was washed with water and brine, before being dried over Na_2SO_4 and concentrated *in vacuo*. The residue was distilled (bp. 92-93 °C at 20 mbar) to give 13 g (86% yield) of 3-methylnon-1-en-4-yn-3-ol (**6**) as colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.97 (dd, J = 17.0, 10.2 Hz, 1H), 5.48 (d, J = 17.0 Hz, 1H), 5.08 (d, J = 10.1 Hz, 1H), 2.23 (d, J = 7.1 Hz, 2H), 1.52 (s, 3H), 1.49 (m, 2H), 1.41 (p, J = 7.3 Hz, 2H), 0.91 (t, J = 7.3 Hz, 2H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 142.6, 113.1, 86.0, 82.2, 67.9, 30.7, 30.2, 21.9, 18.3, 13.6.

2.10.2.b Synthesis of 3-Methyl-2-pentylfuran (3b)

3-Methyl-2-pentylfuran (**3b**) was synthesized from 3-methylnon-1-en-4-yn-3-ol (**6**) in 2 steps using the protocol of Gabriele, et al., (1999). The spectral data were in accordance with the data in the literature.

2.10.2.c Synthesis of 4-Methyl-5-pentylfuran-2-carbaldehyde (8b)

Phosphorous oxychloride (1.21 g, 7.9 mmol) was added dropwise to *N,N*-dimethylformamide (2.5 mL) while stirring at 0 °C. After stirring for 1 h at the same temperature, 3-Methyl-2-pentylfuran (**3b**) (1g, 6.6 mmol) dissolved in 2.5 mL of *N,N*-dimethylformamide was added dropwise while keeping the temperature at 0 °C. After the addition, the reaction was stirred for 1h at room temperature, after which TLC showed complete conversion of the furan. The deep red solution was poured into 5% aqueous NaOH (50 mL) and extracted with diethylether (3 × 20 mL). The extract was washed with water and brine, before it was dried over Na_2SO_4. After evaporating the solvent, the aldehyde was purified by column chromatography (silica gel, 5% EtOAc in hexane) giving 910 mg (77% yield) of 4-methyl-5-pentylfuran-2-carbaldehyde (**8b**). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.46 (s, 1H), 7.04 (s, 1H), 2.65 (t, J = 7.1 Hz, 2H), 2.03 (s, 3H), 1.67 (m, 2H), 1.31 (m, 4H), 0.89 (t, J = 7.0 Hz, 3H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 176.8, 160.0, 150.6, 119.0, 118.0, 31.4, 27.6, 26.4, 22.3, 13.9, 9.7.

2.10.2.d Synthesis of (*E/Z*)-Methyl 9-(4-methyl-5-pentylfuran-2-yl)non-8-enoate (12b)

(7-Carbomethoxyheptyl)triphenylphosphonium bromide (**11**), (3.6g 6.9 mmol) which was synthesized from methyl-8-bromooctanoate (**10**) using the protocol of Tsukasa (1993), was dissolved in 12 mL *N,N*-dimethylformamide under an atmosphere of argon and cooled to 0 °C. Sodium methoxide (470 mg, 8.7 mmol) were added, after which the reaction was stirred for 10 min at the same temperature. After the addition of 900 mg (1.67 mmol) of 4-methyl-5-pentylfuran-2-carbaldehyde (**8b**) the reaction was stirred for 1h at room temperature and then quenched by pouring the mixture on ice. The products were extracted with diethylether (3 × 20 mL) washed with brine and dried over Na_2SO_4. After evaporating the solvent the product was purified by column chromatography (silica gel, 5% EtOAc in hexane) giving 977 mg (61% yield) of a 10:1 mixture of (*E*)- and (*Z*)-methyl 9-(4-methyl-5-pentylfuran-2-yl)non-8-enoate (**12b**). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.07 (dd, J = 11.6, 1.6 Hz, 1H), 6.02 (s, 1H), 5.42 (dt, J = 11.9, 7.3 Hz, 1H), 3.66 (s, 3H), 2.53 (t, J = 7.4 Hz, 2H), 2.39 (qd, J = 12.2, 1.5 Hz, 2H), 2.30 (t, J = 7.5 Hz, 2H), 1.93 (s, 3H), 1.62 (m, 4H), 1.40 (m, 10H), 0.89 (t, J = 7.0, 3H)ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.2, 150.7, 150.4, 129.2, 117.5, 115.2, 112.3, 51.4, 34.1, 31.4, 29.4, 29.2, 29.0, 28.1, 25.9, 24.9, 22.4, 14.0, 9.8. MS (EI, 70eV): *m*/*z* 320 (M^+ 21), 263 (24), 191 (81), 147 (25), 135 (47), 134 (16), 121 (95), 119 (15), 109 (34), 107 (19), 105 (20), 95 (26), 93 (51), 91 (61), 81 (26), 79 (32), 77 (28), 69 (24), 67, 24), 59 (24), 55 (100), 53 (17), 43 (93), 41 (75).

2.10.2.e Synthesis of Methyl 9-(4-methyl-5-pentyl-2-furyl)-nonanoate (13b)

The mixture of (*E*)- and *(Z)*-methyl 9-(4-methyl-5-pentylfuran-2-yl)non-8-enoate (**12b**) (400 mg, 1.25 mmol) was dissolved in 4 mL of methanol and hydrogenated over 10 mg of 10% palladium on activated charcoal at atmospheric pressure.

After 1 h the catalyst was filtered off and the solution concentrated *in vacuo*. Column chromatography (silica gel, 5% EtOAc in hexane) gave 371 mg (92 % yield) of methyl 9-(4-methyl-5-pentyl-2-furyl)-nonanoate (**13b**).

^{1}H NMR (500 MHz,$CDCl_3$) δ 5.72 (s, 1H), 3.66 (s, 3H), 2.51 (t, J = 7.7 Hz, 2H),

2.49 (t, J = 7.5 Hz, 2H), 2.30 (t, J = 7.6 Hz, 2H), 1.89 (s, 3H), 1.58 (m, 4H), 1.30 (m, 12H), 0.89 (t, J = 7.0, 3H) ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.3, 153.4, 149.9, 113.8, 107.7, 51.4, 34.1, 31.4, 29.2, 29.15, 29.1, 28.4, 28.1, 28.0, 25.9, 25.0, 22.4, 14.0, 9.9. MS (EI, 70eV): *m/z* 322 (M^+ 61), 291 (17), 265 (100), 179 (13), 165 (87), 135 (6), 121 (19), 109 (42), 108 (10), 95 (12), 55 (7).

2.10.2.f Synthesis of 9-(4-Methyl-5-pentyl-2-furyl)-nonanoic Acid (14b)

The mixture of (*E*)- and (*Z*)-Methyl 9-(4-methyl-5-pentylfuran-2-yl)non-8-enoate (**12b**) (200 mg, 0.62 mmol) was dissolved in a mixture of methanol (1 mL) and THF (2 mL) and NaOH solution (1.9 mL,1M) were added. After the reaction mixture was stirred for 1.5 h at 60 °C, HCl (2 mL, 1 M) was added and the mixture was extracted with ethyl acetate (3 × 10 mL). The combined organic phase was washed with brine and dried over Na_2SO_4. After evaporating the solvent the acid was purified by column chromatography (silica gel, 20% EtOAc in hexane) giving 175 mg (92% yield) of 9-(4-Methyl-5-pentylfuran-2-yl)nonanoic acid (**14b**). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.72 (s, 1H), 2.51 (t, J = 7.6 Hz, 2H), 2.49 (t, J = 7.5 Hz, 2H), 2.34 (t, J = 7.5 Hz, 2H), 1.89 (s, 3H), 1.60 (m, 4H), 1.30 (m, 10H), 0.88 (t, J = 7.0, 3H)ppm; ^{13}C NMR (125 MHz, $CDCl_3$) δ 179.9, 153.4, 149.8, 113.8, 107.7, 33.8, 31.4, 29.2, 29.1, 29.0, 28.4, 28.1, 28.0, 25.9, 24.7, 22.4, 14.0, 9.9. MS (EI, 70eV): *m/z* 308 (M^+ 30), 252 (17), 251 (100), 166 (7), 165 (48), 134 (16), 121 (10), 109 (17), 108 (5), 107 (19), 95 (5).

3. Results and Discussion

Although the literature does not reveal any evidences so far for the existence of qualitative differences in cuticular fatty acid composition between male and female *D. melanogaster*, our observations on the total ion chromatograms (TIC) of male and female surface lipid extracts which are not identical to each other (Supplementary-1) suggest for the presence of sex dependent nonhydrocarbon components such as fatty acids or fatty acid derivatives.

Still many scientists doubt that the nonhydrocarbon components, particularly triglycerides are not considered as surface lipid components and supposed to be appear when the extraction has been so extensive as to extract internal lipids. It is usually believed that the extraction solvents and the exposure time have been crucial factors that would led to the rupture of cuticle, thus extraction of the internal components (Jackson, et al., 1981). Therefore, extreme care has been taken during the handling and only a superficial rinse of the flies was performed in order to avoid the extraction of any internal lipids.

Since we are interested in investigating the nonhydrocarbon components in the insect surface, our focus was on the medium polar fractions that have been eluted from the silica gel column chromatography. Thus the male and female fractions eluted with 15% EtOAc in hexane (Fra.7) was selected for a in-depth study (**Fig.1**).

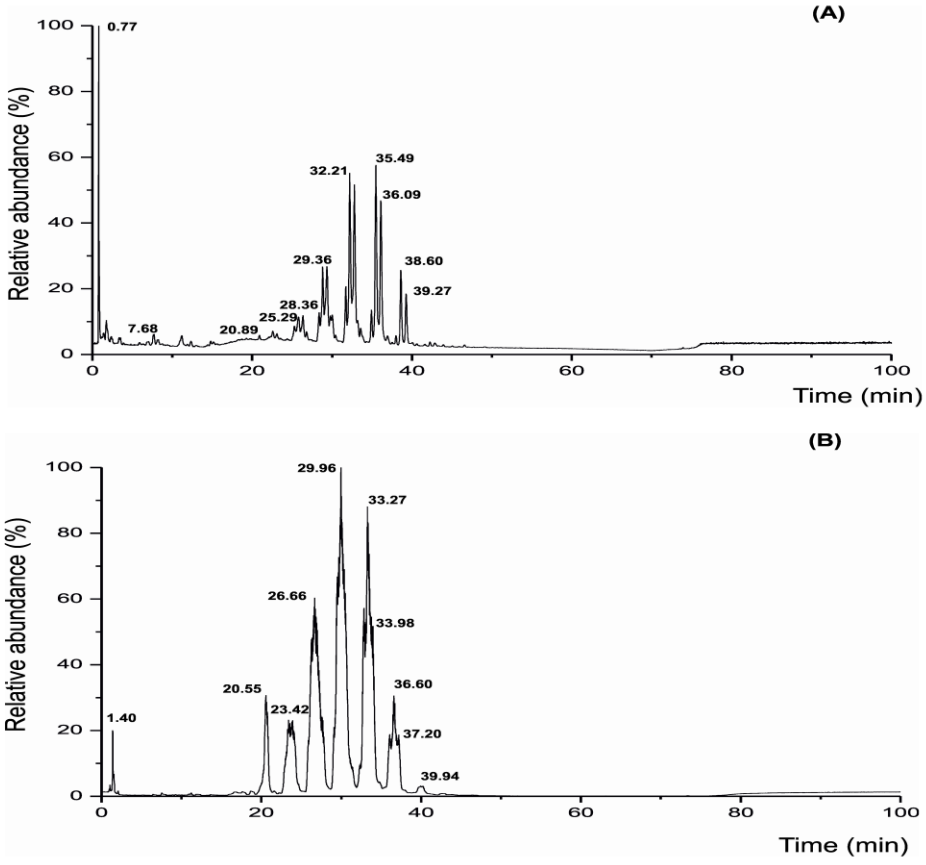


Fig. 1 – UHPLC-APCI Total Ion chromatograms of male (A) and female (B) fraction eluted with 15% EtOAc in hexane

Among the detected peaks, several compounds have exclusively been detected in the female fraction whereas only one compound was detected as male specific. The accurate mass measurements on LTQ Orbitrap XL has been used to determine molecular composition of these unknown compounds. A list of sex specific compounds detected in corresponding female and male fractions eluted with 15% EtOAc in hexane (Fra.7) is given in Supplementary- 2.

Out of the identified compounds, our attention was focused on the compound at *m/z* 963.7275 with the molecular composition of $C_{60}H_{99}O_9$ which presumed to be a TAG. This peak was virtually absent in the corresponding male fraction and it appears interesting as it showed unexpected 19 carbon atoms in the fatty acid chain and nine oxygen atoms. To get more structural insights, CID/HCD experiments have been performed which give rise to one diacylglycerol ion $[M+H-R_iCOOH]^+$ (*m/z* 655.4918), one monoacylglycerol ion $[M+H-R_iCOO-R_i'CO]^+$ (*m/z* 365.2665) and one acyl ion $[R_iCO]^+$ (*m/z* 291.23) (**Fig. 2**). It helped to rationalize the presence of three identical fatty acids with 19 carbon atoms, three double bonds/cycles and additional oxygen in the fatty acid chain.

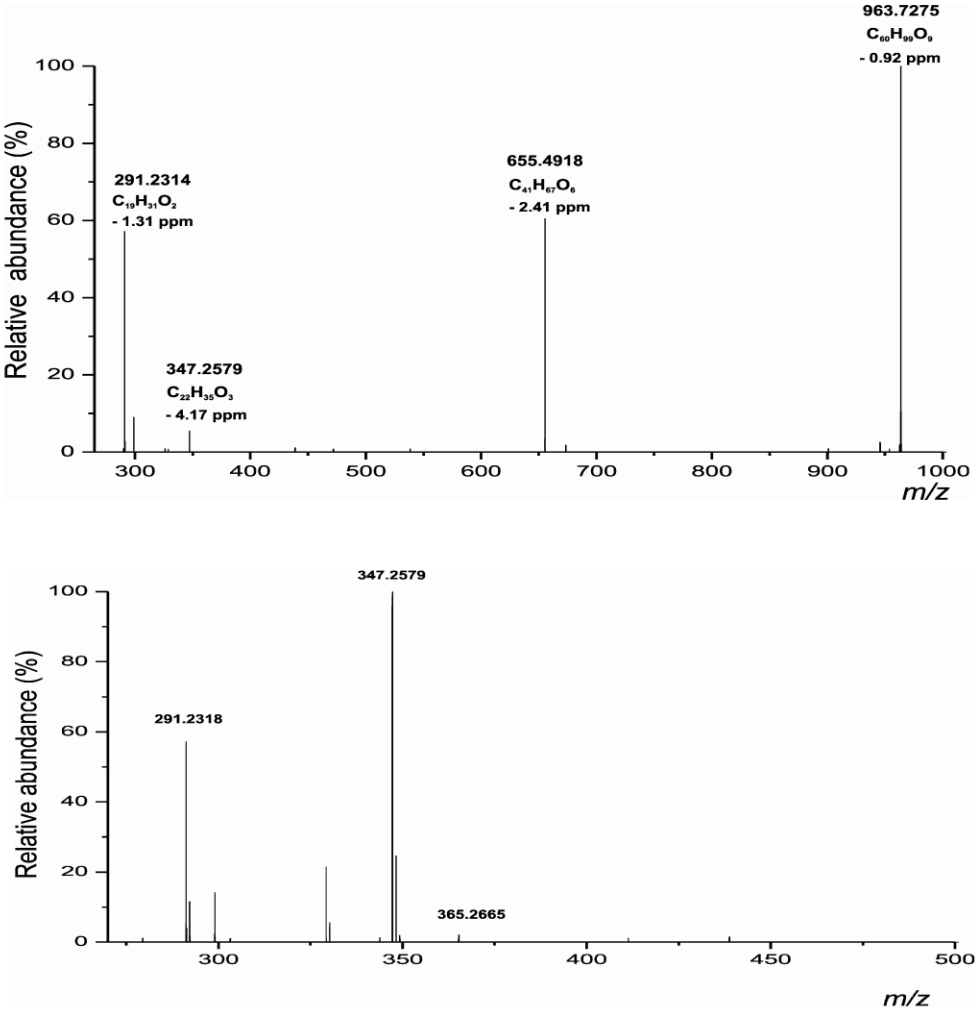


Fig. 2 HCD/CID spectra of the female specific compound of *m/z* 963.7275

In order to confirm the number of double bonds in this molecule, hydrogenation of the intact TAG was performed in which a peak at *m/z* 975.8189 was observed (**Fig. 3**). This corresponds to the hydrogenation of two double bonds in each of the three constituent fatty acids. However the double bond positions were not able to be deduced from the DMDS derivatization, which thus suggests that the double bonds might have not been arranged in a simple aliphatic chain and rather could be in a cyclic form.

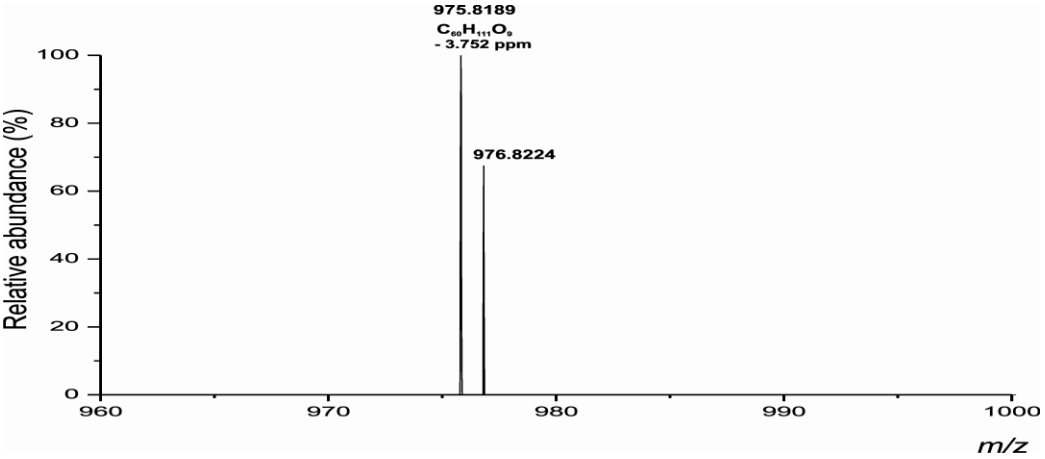


Fig. 3 Peak corresponding to the hydrogenated TAG

In order to determine whether the additional oxygen atom in the fatty acid chain exists as a hydroxyl group or a ketone group, acetylation and oxime formation reactions were performed respectively. However, neither the acetylated product nor the oxime product were detected suggesting the absence of hydroxyl or ketone groups in fatty acid chain.

The accurate mass GC-MS analysis of the trans-methanolyzed TAG provided invaluable details towards the identification of this unusual fatty acid. It has not only supported the previously determined molecular composition but also provided data for partial localization of the desaturation and oxidation. The subsequent data base search in AOCS Lipid Library (William W. Christie, Dundee, Scotland) allowed the identification of this constituent fatty acid as 9-(3-methyl-5-pentylfuran-2-yl)nonanoic acid. The identity of the compound was confirmed by the comparison of GC-MS spectra of the trans-methanolyzed TAG sample with the synthesized fatty acid methyl esters, 9-(3-methyl-5-pentylfuran-2-yl)nonanoate (**13a**) and its' isomer 9-(4-Methyl-5-pentyl-2-furyl)-nonanoate (**13b**) (**Fig.4**).

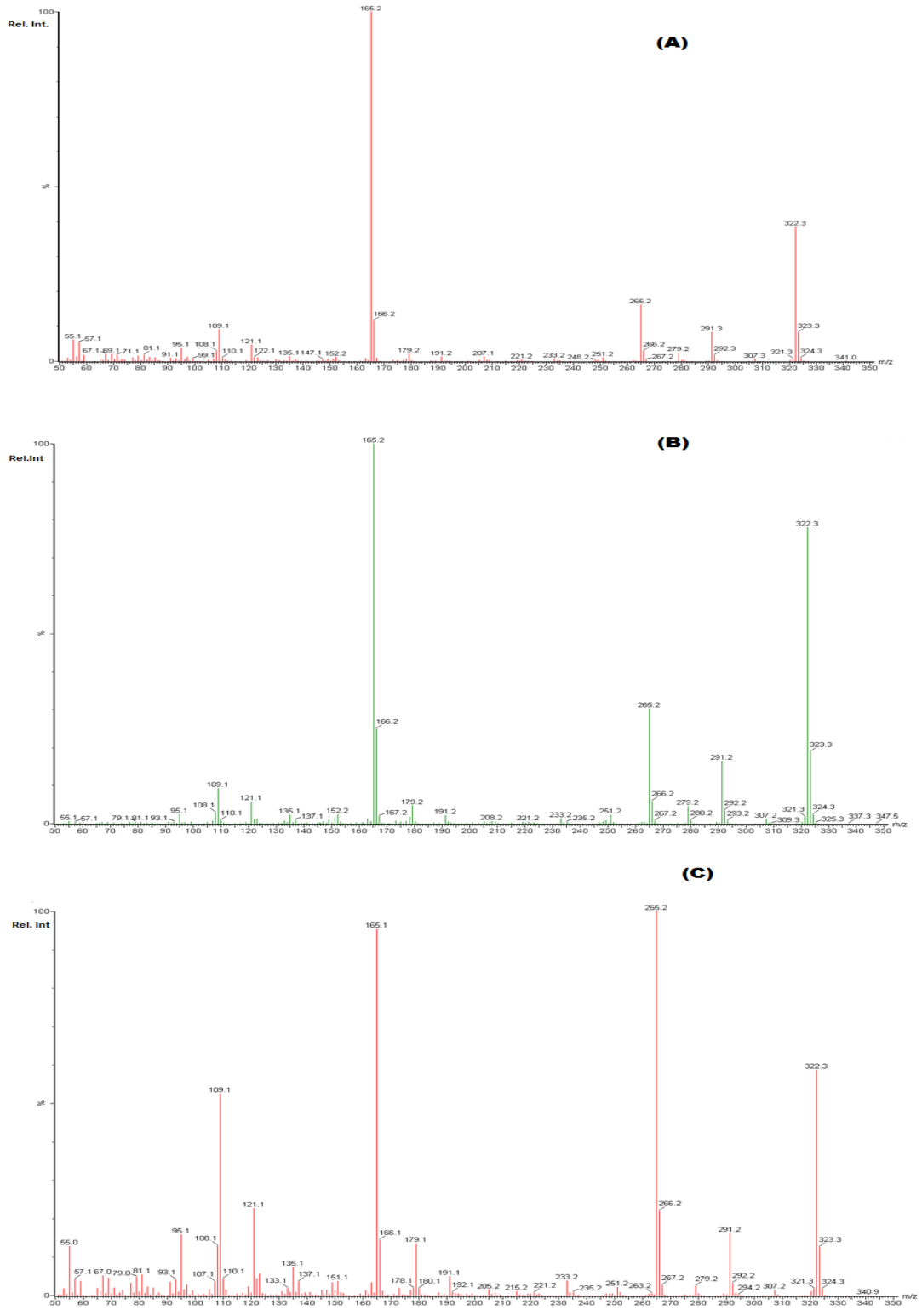


Fig . 4 - GC spectra of (A) -trans methanolyzed TAG, (B)- synthetic 9-(3-methyl-5-pentylfuran-2-yl)nonanoate and (C) 9-(4-Methyl-5-pentyl-2-furyl)-nonanoate

The retention time and the fragmentation pattern of synthesized 9-(3-methyl-5-pentylfuran-2-yl)nonanoate was identical with the trans-methanolyzed TAG, thus confirmed the presence of this complex fatty acid and its TAG as a female specific compound in *D. melanogaster* surface lipid extract.

9-(3-methyl-5-pentylfuran-2-yl)nonanoic acid has been reported in some fish oils and vegetable oils (Vetter, et al., 2012; Guth and Grosch, 1991) previously, however it has never been detected in any insect species to the best of our knowledge. There are reports suggesting that the enteric bacteria in fish are responsible for its synthesis and a putative biosynthetic pathway has been proposed (Shirasaka, et al., 1997) which starts from *cis*-vaccenic acid, a common precursor of the pheromones in *D. melanogaster* (Jallon, 1997). Therefore, the identification 9-(3-methyl-5-pentylfuran-2-yl)nonaoic acid and its TAG form in *D. melanogaster* female flies is of great interest in terms of its biosynthesis as well as the biological significance. Although, we have carried out preliminary experiments with 30 flies of each to track its presence in different development stages (2 day old, 3 day old , 6 day old and 6 day old after mating), the low abundance of the compound in crude extract has hindered its detection. Therefore a large scale extraction of flies of different developmental stages might help to solve this puzzle and hope to be carried out in the future.

Nevertheless, our investigation on sex dependent fatty acids and fatty acid derivatives in *D. melanogaster* led to the findings that contradicts decade's old observations on the absence of qualitative differences between cuticular fatty acid profiles in male and female flies. Further more, it would inspire the scientific community for a detailed study on the biosynthesis and physiological functions of the unusual female specific fatty acid that has been reported for the first time in an insect species.

4. Conclusion

By demonstrating the presence of female specific 9-(3-methyl-5-pentylfuran-2-yl)nonanoic acid and its TAG, the present investigation reveals the existence of qualitative differences between the composition of fatty acid and fatty acid derivatives in male and female *Drosophila melanogaster* flies. This observation contradicts the widely accepted concept of the absence of sex-dependent qualitative differences in the non-hydrocarbon components in the cuticle of *Drosophila melanogaster* and inspired for further study on the biosynthesis and physiological functions of these sex specific compounds.

Acknowledgements

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Supplementary – 1

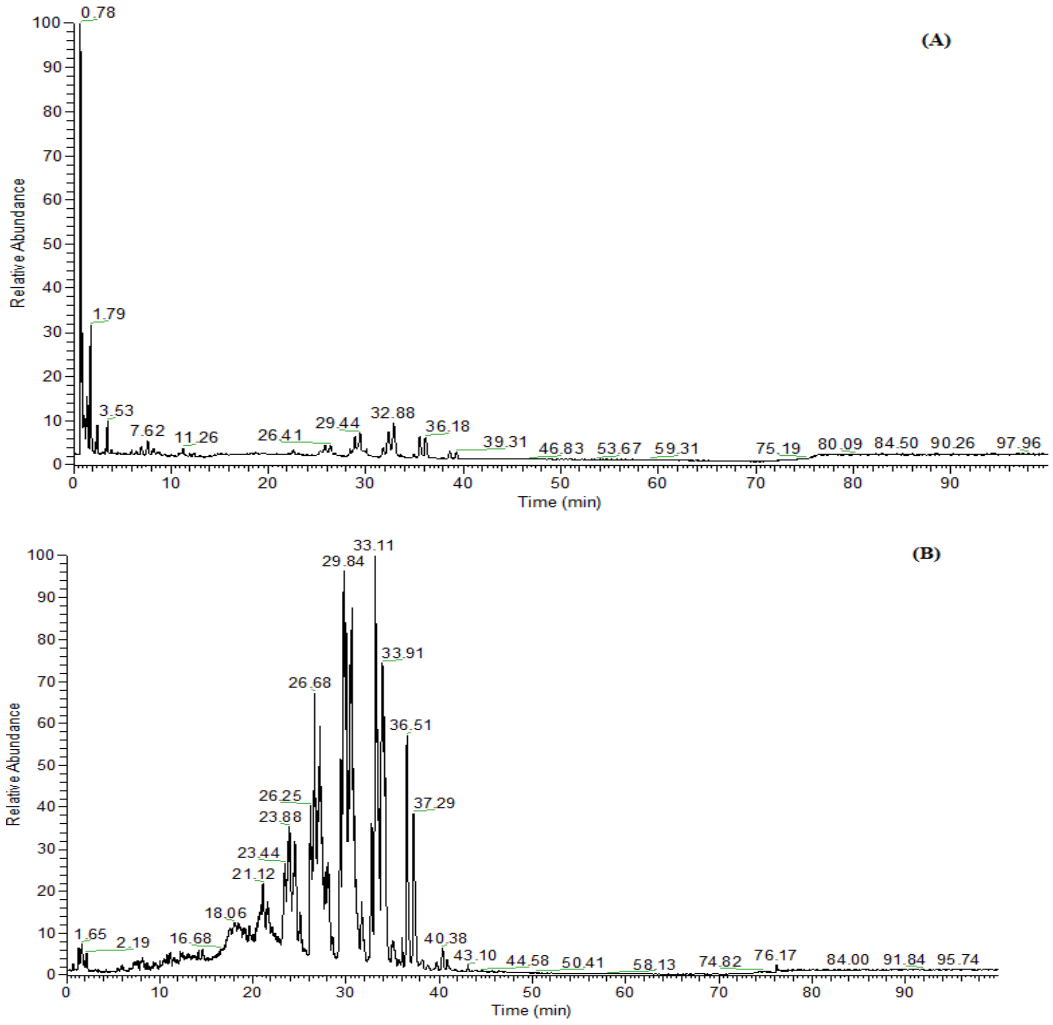

Fig S-1. UHPLC-APCI Total Ion Chromatograms of male (A) and female (B) surface lipid crude extracts

Supplementary – 2

Sex dependent compounds in corresponding male and female fractions eluted with 15%EtOAC in hexane (Fra.7) in the silica gel column chromatography

Compounds present only in the Female Fra.7

m/z	Intensity	Rt(min)	Possible composition	Δ ppm
768.7082	5.01 E^5	33.82	$C_{47}H_{84}O_5N$	0.812
935.7342	3.70 E^5	24.69	$C_{55}H_{99}O_8$	1.126
			$C_{62}H_{97}O_5N$	-1.802
963.7275	8.21 E^6	23.42	$C_{60}H_{99}O_9$	0.922
1018.77	4.74 E^5	23.11	$C_{60}H_{106}O_{12}$	1.737

Compounds present only in the Male Fra.7

m/z	Intensity	Rt	Possible composition	Δ ppm
546.5231	1.33 E^5	13.07	$C_{33}H_{70}O_5$	2.366
			$C_{36}H_{68}O_2N$	-2.538

Chapter 6

Discussion

Discussion

A few decades ago, the task of mass spectrometry was limited to providing information on elemental composition of a molecule and its partial structural insights and has been seldom used for the *de novo* structural characterization of small molecules (Kind and Fiehn, 2010). However, with the recent advancements in instrumentation, the roles of mass spectrometry have been widely expanded and need to be re-defined. Today it is considered as an indispensable tool for structural characterization of secondary metabolites in the field of natural products chemistry.

The present study, which has been undertaken, under three themes as mentioned in Section 1.4, is an attempt to exploit mass spectrometry in the analysis of diverse forms of secondary metabolites. This is achieved by optimizing the existing mass spectrometric tools while developing high throughput analytic methods for studies of natural products and metabolomics.

The efforts we have made in this thesis to explain the development and optimization of mass spectrometric approaches for the analysis of secondary metabolites in a wide array of biological samples clearly reflects the expediency of mass spectrometry in "small molecule research". In addition, the chemistry and potential bioactivities of the secondary metabolites that have been characterized in this study will undoubtedly be topics of interests for many researchers in the future.

The significance of our mass spectrometric approaches and the contribution of our findings on secondary metabolites for prospective applications in diverse fields are discussed below.

Novel MALDI matrices for metabolomics studies

Until recently, MALDI-MS has been extensively used in the analysis of large biomolecules like proteins, peptides, oligosaccharides etc. (Karas and Hillenkamp, 1988) and pigeonholed as a "high-molecular mass tool only". The high sensitivity and high throughput nature of this technique made it to be a good choice for such analysis (Karas, et al., 1987). The success in the analysis of a wide variety of molecules lies in its ability to generate intact ions of thermally labile molecules using UV absorbing matrices. A good matrix should be able to absorb light at the wavelength of the laser used and several molecules with this property have been employed in the analysis of large biomolecules (Cohen, et al., 2007). As most of these conventional matrices have a molecular weight below 500 Da, the presence of matrix related ions in the low mass region of the spectrum was a common phenomenon. Since all analytes of interest are in the high-mass range, this forest of interfering peaks produced by the conventional matrices in the low mass region was not an issue of concern.

During the last decade, MALDI-MS has gained much attention in the arena of small molecule research due its incredible tolerance to salts and buffers, minimal requirements for sample preparation or pretreatment and the relative ease of spectral interpretation. However, the application has been suppressed mainly due to the unavailability of suitable matrices (Cohen, et al., 2007).

A careful selection of matrix is a crucial factor in successful application of MALDI to small molecule analysis and a compound that could provide an efficient ionization, minimal or controllable fragmentation and lack of mass interferences is considered to be an ideal matrix. However, due to the lack of knowledge on overall mechanisms of the processes of desorption and ionization, the choice of MALDI matrices has remained empirical.

To address this issue, a novel tactic for the selection of "ionless" matrices was introduced by our laboratory (Shroff, et al., 2009). It is based on the Brønstead-Lowry acid-base theory. Two novel matrices 1,8-bis(dimethylamino)naphthalene (DMAN)

and 2-naphthylsulfonic acid were developed for the negative and positive mode MALDI analysis respectively, which give rise to spectra completely devoid of any matrix-related ions.

A new mode of ionization, Matrix Assisted Ionization/Laser Desorption (MAILD) was suggested as the mode of ionization when either a strong base (DMAN) or a strong acid (2-naphthylsulfonic acid) is used as a matrix in the above situations. In the analysis of acidic metabolites in the negative ion mode, UV absorbing super base matrix DMAN is premixed with an acidic analyte. The ionization takes place in the solution during the mixing of a weak to moderate acidic analyte with the strong base matrix. Proton exchange from analyte to matrix results in the formation of salt/ion pair on the MALDI target plate that is desorbed by the laser pulses and separated into "naked ions" in the gas phase.

However, it was recently realized that under experimental conditions, DMAN is volatile and may desorb deposits in MALDI/TOF-MS instruments' ion sources, thus possibly yielding interfering peaks in the mass spectra (Eibisch, et al., 2012). In order to eliminate this problem and to enhance the capability of MALDI-TOF-MS in low molecular weight compound analysis, we have been extensively searching for alternate MAILD matrices either by modification of DMAN or looking in other classes of super bases. Manuscript-I summarizes our success in this endeavor.

A new class of "proton sponges", named azahelicenes were tested in this study. Some of these azahelicenes have displayed exceptional basicities (Roithová, et al., 2007: Staab, et al., 1994) even though these compounds lack hydrophobic shielding in their basic centers and N---H---N hydrogen bridges, which is considered as the characteristic feature of all classical proton sponges (Staab and Saupe, 1988). Not surprisingly, out of the tested azahelicenes, an exceptional performance by 1,14-diaza[5]helicene as a MAILD matrix was observed, which was further supported by the measured/calculated physiochemical parameters.

The higher gas phase proton affinity, higher pK_a and higher pK_b for deprotonation values observed for 1,14-diaza[5]helicene, as well as the absorbance maximum in the frequency close to that of the lasers used, strongly favor a MAILD type of ionization when it is mixed with an acidic analyte and leads to the formation of a conjugated acid/base ion pair. Upon UV laser irradiation, the ion pair absorbs light energy and effectively desorbed and a clear spectrum without any matrix related peaks could be visible for the deprotonated analyte.

A schematic illustration of the MAILD ionization is given in **Fig.3.1**

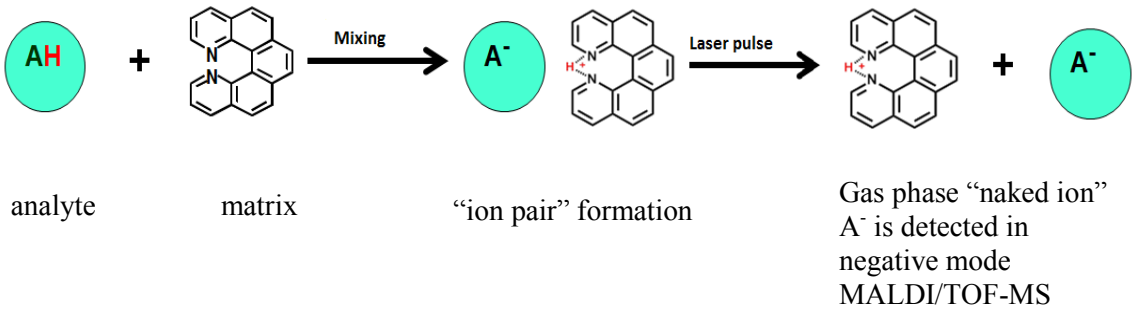

Fig. 6.1 Suggested mechanism of MAILD type ionization for 1,14-diaza[5]helicene

The successful application of 1,14-diaza[5]helicene in the analysis of organic acids in fruit juice and wine samples (Manuscript-I, fig.1 and Suppl. fig.3) further suggests the feasibility of using of this matrix in quantification of organic acids with the use of stable isotopes, which is one of the current topics of interest in our laboratory. This would open up a relatively untouched area in mass spectrometry where MALDI-MS is rarely employed in quantification of small molecules, and thus address the ever-present demand for a high throughput analytical tool for quantification in the pharmaceutical arena.

Therefore, our findings on novel MALDI matrices for small molecule analysis not only demonstrate the versatility of MALDI-MS beyond its conventional uses in high-molecular weight compound analysis, but also open up new venues for high throughput metabolomics.

Identification of bioactive secondary metabolites from medicinal plants in Sri Lanka

Although, the flora of Sri Lanka is enriched with a vast number of biologically active secondary metabolites which have been investigated for antimicrobial, insecticidal, piscicidal, immunomodulatory etc. properties (Hewage, et al., 1997; Hewage, et al., 1998), a large portion of plants still remains unexplored. Plants and their products have been extensively used in indigenous medicine and most of the domestic supply of plants comes from the wild, causing overharvesting of populations from their natural habitats. In addition, increased demand for agricultural land and unsustainable cultivation practices are destroying the natural habitats of medicinal plants and as a result some of the medicinal plants (eg. *Munronia pinnata*) are in the threat of being endangered (Wijesundera, 2004). Even though, isolation and structural elucidation of new natural products from these medicinal plants and investigation of their bioactivities is rewarding, the collection of plant material for conventional natural product isolation strategies is becoming unfeasible due to the scarcity of plants. In addition, putting more efforts into isolating each compound in pure form by performing tedious multi-step separation and purification processes are becoming less attractive since these approaches are no longer cost-effective and time-effective. Thus, the emergence of online characterization methodologies of secondary metabolites in crude natural product extracts or fractions has provided a solution to these long persisting problems and made life easier for natural product chemists.

Online characterization of secondary metabolites in crude natural product extracts or fractions demands high degree of sophistication, richness of structural information, sensitivity, and selectivity. The remarkable improvements in hyphenated analytical methods offer shorter analysis time, higher degree of automation, higher sample throughput, better reproducibility, enhanced selectivity and therefore a higher degree of information (Joshi, et al., 2012). In particular, the addition of Orbital trap mass analyzer to the arsenal of mass spectrometric analyzers supports a wide range of

applications from routine compound identification to the analysis of trace-level components in complex mixtures, permitting the characterization of natural products directly from the crude extract with a minimal amount of material (Perry, et al., 2008; Hu, et al., 2005).

Manuscripts-II and III summarize our achievements in the application of MS-based rapid screening strategies for characterization of bioactive metabolites in two popular medicinal plants in Sri Lanka that are neither phytochemically nor pharmacologically explored yet.

Twelve medicinal plants were initially selected for this study based on their use in the traditional system of medicine in Sri Lanka and only a few grams (maximum 20 g) of plant materials were used in the preparation of crude extracts which were then subjected to several bio-assays such as 5-lipoxygenase inhibition, microsomal prostaglandin E_2 synthase-1 inhibition, cell viability of human leukemic cells, antioxidant and reactive oxidant scavenging assays. Out of the tested crude extracts, the most active four extracts were subjected to partial fractionation by silica gel column chromatography and the resulted fractions were tested for the 5-lipoxygenase inhibitory activity. The most active fractions that exhibited 5-lipoxygenase inhibition at a concentration of 1μg/mL were thoroughly analyzed to identify the presence of possible bioactive compounds.

In this investigation, UHPLC system coupled to LTQ Orbitrap XL instrument and GC-MS have been employed as sole techniques in structural characterization of secondary metabolites. The accurate mass measurements of adduct ions by the Orbitrap instrument enabled the determination of molecular composition within 1-5 ppm mass errors and database searching of exact masses for possible relevant secondary metabolites. MS/MS data has provided powerful tool to dereplicate possible structures. To obtain more clear overview, the fragments resulted from MS/MS experiments were further analyzed by computer assisted algorithms to yield hypothetical fragmentation trees which allow the assignment of specific relevant fragments and fragmentation pathways.

As emphasized in Manuscript-II, the traditional use of the Sri Lankan endemic plant *Plectranthus zeylanicus* for the treatment of inflammatory conditions could be rationalized with our phytochemical findings. In addition to the identification of several known compounds that have been already studied for their anti-inflammatory activities, the detection of coleone P in the active fraction has immense significance. This is not only because of its occurrence in this plant is reported for the first time, but also due to the high possibility of this compound to exhibit anti-inflammatory or anti-proliferative properties. Since coleon variety of diterpenoids which are characteristic to genus *Plectranthus* have already demonstrated several bioactivities including antiproliferative activity (Marques, et al., 2002; Xing et al., 2008), a detailed study on the bioactivities of coleone P would definitely inspire the pharmacological community.

Manuscript-III describes our scientific investigations on one of the most valued and rare medicinal plants in Sri Lanka, which has never undergone thorough phytochemical analysis, despite the numerous efforts made over the years. This first ever report on the chemical profile of popular Sri Lankan folk medicinal plant *Munronia pinnata*, would not have been possible without the advanced hyphenated techniques, as its phytochemical investigations have been severely hampered by the dearth of plant material for traditional natural product extraction strategies. The conventional use of *M. pinnata* in Sri Lankan indigenous medicine for the treatment of fever and several other inflammatory conditions (Department of Ayurveda, 1979; Jayaweera, 1982) was supported by our chemical profiling. It has revealed the presence of several secondary metabolites with already known anti-inflammatory activities.

Although the emphasis in this phase of the study was mainly on the identification of known bioactive metabolites, so as to validate the long-established use of plants in Sri Lankan folk medicine, there's a great possibility to extend this study towards the identification of unknown compounds in the active extracts and fractions. Even though these compounds are not in any database, the recently introduced automated

alignment of fragmentation trees (FT-BLAST, fragmentation tree basic local alignment search tool) could be utilized for the identification process (Rasche, et al., 2012). The computed fragmentation trees which have already been employed in this study for the compound identification provided a good explanation of our observed data, thus, we expect FT-BLAST approach to be a promising tool in the identification of unknowns in the active extracts and fractions.

Therefore, our MS based rapid screening of plant extracts not only substantiates the conventional use of medicinal plants in Sri Lanka but also proves that phytochemical screening is no longer as cumbersome as it was a few years before.

Identification of sex dependent lipids in *Drosophila melanogaster*

The cuticular chemistry of insects has become a topic of interest with the realization of its involvement in various types of chemical communications (Howard, 1993). Normally, the insect cuticle is covered with complex mixtures of nonpolar and polar compounds out of which hydrocarbons comprise the majority while short-chain unsaturated aldehydes, ketones, fatty acids, and acetate esters of short-chain unsaturated alcohols are also present as minor components (Blomquist and Jackson, 1979).

There are several MS based techniques widely employed in the analysis of insect surface lipids. GC-MS is a well-established tool for the detection and structure elucidation of sufficiently volatile nonpolar cuticular hydrocarbons, however larger and more polar cuticular compounds are likely to be missed by this method. Therefore the conventional approach generally relied on hydrolysis of lipids followed by derivatization in to fatty acid methyl esters (FAME) and subsequent GC-MS analysis of released fatty acid derivatives. However, the analysis of FAMEs by GC-MS does not provide complete information about the original lipid molecule, especially how the constituent FAs were bonded to the glycerol backbone. In the case

of unsaturated FAs, the double bonds tend to migrate along the aliphatic hydrocarbon chains in the electron ionization mode, challenging the localization of double bond (Mossoba, et al. 1994). Therefore, different methods like analysis of dimethyl disulfide (DMDS) adducts (Dunkelblum, et al., 1985), positive-ion chemical ionization using acetonitrile as reactant gas in GC-ion trap (Moneti, et al., 1996; Oldham and Svatoš, 1999; Van Pelt and Brenna, 1999; Kroiss, et al., 2011) were introduced for the analysis of unsaturated lipids and localization of their double bonds.

The emergence of HPLC/APCI-MS as a novel tool offers advantages over GC-MS in the analysis of complex lipid mixtures. The analysis of triacylglycerols (TAG) of higher molecular weight (high equivalent carbon number-ECN) was achieved by optimizing non-aqueous reversed phase HPLC on octadecyl-modified silica (Kofroňová, et al., 2009) and successfully applied in the analysis of insect TAG (Cvačka, et al., 2006 a). APCI affords structure related fragment ions in the first MS step. The interpretation of the spectral data has greatly sped up with the development of "TriglyAPCI", an algorithm for automatic APCI mass spectra interpretation, which uses diacylglycerol fragments and molecular adducts to suggest the structures of TAG while precluding possible mistakes (Cvačka, et al., 2006 b). All these developments significantly contribute in the analysis of even trace components in complex surface lipid extracts, thus uncovering the hidden chemistry of the insect cuticle.

Manuscript-IV describes our achievements in studying the sex-dependent surface lipids in *Drosophila melanogaster* with the help of UHPLC-APCI-MS and GC-MS techniques that shed new light onto the field of *Drosophila melanogaster*'s cuticular chemistry.

The observations on sex dependent differences in composition of cuticular lipids in several Diptera species, have prompted the investigations of surface lipids in *D. melanogaster* and since then several cuticular hydrocarbons that display sexual dimorphism and perform pheromonal functions have been identified (Jallon, 1984 ;

Ferveur, 2005; Everaerts, et al., 2010). However, the attention paid to other types of cuticular substances is not sufficient and until now only a handful of investigations have been conducted to study the non-hydrocarbon components especially fatty acids and fatty acid derivatives. With quantitative differences existing between them, myristic acid, palmitic acid, palmitoleic acid, oleic acid and linoleic acid were identified as major fatty acids from both the acylglycerol and free fatty acid fractions of both sexes. It was observed that there were no significant differences between the fatty acid compositions of either free fatty acids or acylglycerol fatty acids from male and female flies (Jackson, et al., 1981) and no evidence has been reported so far to disprove this observation.

However, this concept was challenged with the identification of female specific 9-(3-methyl-5-pentylfuran-2-yl)nonanoic acid and its TAG form, as described in the Manuscript-IV. Even though this triglyceride is present in trace amount in female surface lipid extract, the LC-APCI-MS enabled its detection and even purification with UHPLC system with a gradient of acetonitrile and propan-2-ol as mobile phases (Manuscript-IV, fig.1 and fig.S1). This peak was virtually absent in samples from males of the same age. Since purification yielded only a low amount of the compound, the NMR data was not sufficient to get the structural details of this complex molecule. Therefore GC-MS analysis has played the key role in characterization of its structure.

The accurate MS and MS/MS spectra of the intact TAG obtained by APCI ionization on LTQ Orbitrap XL has been used to determine molecular composition of the unknown TAG. It showed unexpected 19 carbon atoms in the fatty acid chain and nine oxygen atoms. CID experiment has given rise to one diacylglycerol ion $[M+H-R_iCOOH]^+$, one monoacylglycerol ion $[M+H-R_iCOO-R_i'CO]^+$ and one acyl ion $[R_iCO]^+$ (Manuscript-IV, fig.2). It was rationalized that the three identical fatty acids with 19 carbon atoms, three double bonds/cycles and an additional oxygen in the fatty acid chain. The accurate mass GC-MS analysis of the trans-methanolyzed TAG has supported previously determined molecular compositions and provide data for partial

localization of the desaturation and oxidation. Subsequent database search has allowed the identification of the constituent fatty acid and thereby well demonstrated the capacity of mass spectrometry in structural characterization of complex molecules that are present in trace amounts.

Although, 9-(3-methyl-5-pentylfuran-2-yl)nonanoic acid has been reported in some fish oils and vegetable oils (Vetter, et al., 2012 ; Guth and Grosch, 1991) it has never been detected in any insect species to the best of our knowledge. There are reports suggesting that the enteric bacteria in fish are responsible for its synthesis and a putative biosynthetic pathway has been proposed (Shirasaka, et al., 1997) which starts from *cis*-vaccenic acid, a common precursor of the pheromones in *D. melanogaster* (Jallon,et al., 1997). Therefore, the identification 9-(3-methyl-5-pentylfuran-2-yl)nonanoic acid and its TAG form in *D. melanogaster* female flies would instigate the scientific community to conduct investigations on its biosynthesis as well as its biological significance.

Therefore, our mass spectrometric approach in identification of sex dependent surface lipids in *D. melanogaster* not only reveals the presence of female specific 9-(3-methyl-5-pentylfuran-2-yl)nonanoic acid and its TAG and hence the existence of qualitative differences in cuticular fatty acid profiles in male and female flies, but also highlights the necessity of a detailed study on the biosynthesis and physiological functions of this female specific fatty acid.

Conclusions

This thesis reflects the capacity of mass spectrometry in the analysis of secondary metabolites in complex mixtures with the development and optimization of high throughput analytic methods.

The success in development of novel MALDI matrices facilitates the study of low molecular weight metabolites by MALDI-MS and directs towards the exploration its

possibilities to employ in high throughput quantifications. The characterization of secondary metabolites in two Sri Lankan medicinal plants which are still regarded as phytochemically unexplored, by UHPLC-Orbitrap in combination with the fragmentation trees as well as by GC-MS analysis contributes to rationalizing the traditional use of these plants, while promising to pharmacologists much room for more detailed bioactivity studies. The identification of the female specific fatty acid and the TAG in *Drosophila melanogaster* by UHPLC-APCI-MS and GC-MS analysis contradicts the decades-old observations on the absence of qualitative differences in cuticular profiles of male and female flies and has inspired a detailed study of its biosynthesis and physiological functions.

In short, the development and optimization of high throughput and efficient approaches turns mass spectrometry into a powerful workhorse for the analysis of nature's secrets.

Chapter 7

Summary

Summary

Over the years, mass spectrometry has increasingly become an analytical tool of choice in the field of natural products chemistry owing to its high throughput nature, quantitative capability and the facility to integrate with chromatographic separation methods. This study enhances the capacity of mass spectrometry in the analysis of wide variety of complex mixtures with the development of novel MALDI matrices as well as optimizing the existing hyphenated techniques, and thereby cast new light on some pharmacological and ecological aspects that have not been successfully addressed yet. The initial success in development of 1,14-diaza[5]helicene as a novel MALDI matrix for the analysis of acidic analytes demonstrates the applicability of MALDI-MS in small molecule analysis and challenges the perception of MALDI-MS to be a "high molecular mass tool only". Furthermore, it confirms the recently introduced strategy for the selection of "ionless" matrices based on the Brønsted-Lowry acid-base theory and signifies the importance of rational matrix design. These findings indicate a tremendous potential for studying biological systems and opens up new venues for high throughput targeted metabolomics work. Apart from MALDI-MS, the thesis demonstrates the immense contribution of hyphenated techniques in the study of secondary metabolites as indicated in the chemical profiling of two phytochemically unexplored medicinal plants in Sri Lanka. In comparison to the extracts of other medicinal plants reported in literature, the lipophilic extracts of *Plectranthus zeylanicus* and *Munronia pinnata* have exhibited extremely potent 5-lipoxygenase inhibitory activity suggesting a high pharmacological potential of these plants for intervention with 5-LO related disorders. This observation was reinforced by UHPLC-ESI-MS and GC-MS analysis of the active extracts and fractions, which have revealed the presence of several bioactive secondary metabolites together with some compounds for which the knowledge regarding bioactivities are rare. In particular, the identification of coleone P for the first time in *P. zeylanicus*, would spotlight the pharmacological community for future comprehensive studies, due to

the high possibility of this compound to exhibit anti-inflammatory or anti-proliferative properties. The hidden phytochemistry in *M. pinnata* has been unveiled for the first time and the platform laid by this study will be indispensable for further phytochemical and bioactivity research on this precious and rare medicinal plant in future. Therefore, the MS-based rapid screening of medicinal plants substantiates the traditional use of these plants as anti-inflammatory remedy, while ensures that phytochemical screening is no longer as cumbrous as it was a few years ago. The applications of novel hyphenated techniques could be expanded beyond the discovery of pharmaceuticals, as reflected by the study on complex surface lipids extracts in *Drosophila melanogaster*. The identification of female specific 9-(3-methyl-5-pentylfuran-2-yl)nonanoic acid and its TAG by UHPLC-APCI-MS and GC-MS analysis contradicts the decades-old concept of the absence of qualitative differences between cuticular fatty acid profiles in male and female flies. Despite its occurrence in several other biological sources, the biosynthesis of this fatty acid is not clear yet, however, it was suggested that the putative biosynthetic pathway starts from *cis*-vaccenic acid, a common precursor of the pheromones in *D. melanogaster*. Therefore, the detection of this female specific fatty acid for first time in an insect would have a great significance and inspire the biologists for detailed study of its biosynthesis and physiological functions.

In conclusion, the thesis breaks new grounds in several aspects of natural product chemistry with the development and optimization of high throughput, efficient and robust mass spectrometric approaches.

Zusammenfassung

In den letzten Jahren wurde die Massenspektrometrie zunehmend zum analytischen Werkzeug der Wahl im Bereich der Naturstoffchemie, dank ihres hohen Potentials in Bezug auf Probendurchsatz und Quantifizierung, sowie die einfache Integrierbarkeit in chromatografische Trennmethoden. Diese Arbeit erweitert die Möglichkeiten der Massenspektrometrie in der Analyse einer Vielzahl von komplexen Stoffgemischen einerseits durch die Entwicklung neuartiger MALDI-Matrixverbindungen und andererseits durch die Optimierung bereits bestehender Techniken. Sie beleuchtet dabei einige pharmakologische und ökologische Sachverhalte, die bisher nicht erfolgreich aufgeklärt wurden. Der anfängliche Erfolg bei der Entwicklung der 1,14-Diaza[5]helicene als einer neuen MALDI-Matrix zur Analyse acider Verbindungen, zeigt die Anwendbarkeit von MALDI-MS in der Untersuchung von kleinen Molekülen und stellt somit die vorherrschende Ansicht, dass MALDI-MS nur „ein Werkzeug für hochmolekulare Verbindungen“ sei, in Frage. Desweiteren bestätigt er die kürzlich eingeführte Auswahlstrategie für „ionenlose“ Matrices gemäß der Säure-Base-Theorie nach Brønsted und Lowry und unterstreicht die Wichtigkeit eines rationellen Vorgehens bei der Entwicklung von Matrices. Diese Ergebnisse deuten ein gewaltiges Potential zur Untersuchung biologischer Systeme an und eröffnen neue Wege für metabolomische Studien mit hohem Durchsatz.

Neben der MALDI-MS veranschaulicht die vorliegende Arbeit den immensen Beitrag, den gekoppelte Analysemethoden zur Aufklärung von Sekundärmetaboliten liefern. Dies wird im Erstellen des chemischen Profils von zwei phytochemisch unerforschten Heilpflanzen aus Sri Lanka gezeigt. Im Vergleich zu Extrakten aus anderen dokumentierten Heilpflanzen weisen die lipophilen Extrakte aus *Plectranthus zeylanicus* und *Munronia pinnata* extreme Aktivität zur Inhibierung der 5-Lipoxygenase auf und legen damit ein hohes pharmakologisches Potential zur Behandlung von Krankheiten nahe, die mit 5-LO in Zusammenhang stehen. Diese Beobachtungen wurden durch UHPLC-ESI-MS und GC-MS-Analysen der aktiven

Extrakte und Fraktionen bekräftigt, welche das Vorhandensein von mehreren bioaktiven Sekundärmetaboliten enthüllte, zusammen mit einigen Verbindungen, über deren Bioaktivität kaum etwas bekannt ist. Insbesondere der erstmalige Nachweis von Coleon P in *P.zeylanicus* wird Pharmakologen zu umfassenden Studien bewegen, aufgrund der hohen Wahrscheinlichkeit, dass diese Verbindung anti-inflammatorische oder anti-proliferative Eigenschaften besitzt. Die verborgene Phytochemie in *M. pinnata* wurde erstmalig enthüllt und somit ein Grundstein gelegt, der für zukünftige phytochemische Untersuchungen bezüglich der Bioaktivität dieser seltenen und wertvollen Heilpflanze unverzichtbar sein wird. Das MS-basierte schnelle Screening dieser Heilpflanzen erklärt daher nicht nur ihre traditionelle Verwendung zur Entzündungshemmung, sondern zeigt auch, dass phytochemisches Screening nicht mehr so aufwendig und zeitraubend ist wie noch vor ein paar Jahren.
Die Anwendbarkeit von neuartigen gekoppelten Methoden konnte über die Entdeckung von Arzneimitteln hinaus erweitert werden, wie die vorliegende Studie über komplexe lipidhaltige Oberflächenextrakte von *Drosophila melanogaster* zeigt. Mittels UHPLC-APCI-MS und GC-MS konnte 9-(3-Methyl-5-pentylfuran-2-yl)-nonansäure und ihr entsprechendes Triacylglycerid nachgewiesen werden, jedoch nur bei weiblichen Individuen. Damit konnte das über Jahrzehnte bestehende Konzept widerlegt werden, welchem zufolge es keine qualitativen Unterschiede zwischen der Zusammensetzung cutikularer Fettsäuren zwischen männlichen und weiblichen Fruchtfliegen gibt. Obwohl diese Verbindung auch aus anderen biologischen Quellen bekannt ist, ist deren Biosynthese nicht vollständig aufgeklärt. Jedoch wird angenommen, dass der Biosyntheseweg von *cis*-Vaccensäure ausgeht, einem typischen Pheromonvorläufer in *D. melanogaster*. Die erstmalige Entdeckung dieser weibchenspezifischen Fettsäure hat daher eine große Bedeutung und wird Biologen zu detaillierten Studien bezüglich ihrer Biosynthese und ihrer physiologischen Funktion anregen. Abschließend lässt sich sagen, dass die Arbeit durch die Entwicklung und Optimierung von effizienten und zuverlässigen massenspektrometrischen Methoden in einigen Bereichen der Naturstoffchemie neue Wege einschlägt.

Chapter 8

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(for Introduction and Discussion sections)

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Appendices

Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Jena, den 16. September 2014

Mayuri Tharanga Napagoda

Erklärung über laufende und frühere Promotionsverfahren

Hiermit erkläre ich, dass ich keine weiteren Promotionsverfahren begonnen oder früher laufen hatte. Das Promotionsverfahren an der Biologisch-Pharmazeutischen Fakultät ist mein erstes Promotionsverfahren überhaupt.

Jena, den 16. September 2014

Mayuri Tharanga Napagoda

Curriculum Vitae

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2. Educational Background

- March 2010 onwards: **PhD student** at Friedrich-Schiller-University and Max Planck Institute for Chemical Ecology, Jena, Germany.
  Dissertation Title: Metabolomics for Natural Products: Fast screening and Discovery

- 2002- 2005 : **Master of Philosophy** - University of Peradeniya, Sri Lanka
  Dissertation Title: Bioactivity studies of some Sri Lankan flora and bioactive Xanthones from *Calophyllum thwaitesii*

- 2003- 2004 : **Postgraduate Certificate** Course in Advanced Organic Chemistry, University of Peradeniya, Sri Lanka

- 1997-2001 : **Bachelor of Science (Special Degree -Botany)**- First Class Honours, University of Colombo, Sri Lanka
  Dissertation Title: Investigation of characteristics of isolates of *Rigidiporous microporous*, the causative fungus of the white root disease

3. Work Experiences

- June 2005- To date: **Lecturer in Biochemistry**, Faculty of Medicine, University of Ruhuna, Sri Lanka

- July 2002- May 2005: **Research Assistant**, Institute of Fundamental Studies, Kandy, Sri Lanka.

- December 2001- June 2002: **Teaching Assistant in Botany**, Department of Plant Sciences. University of Colombo, Sri Lanka

4. Language Proficiency

English - Very Good (IELTS Overall Band Score of 7.5)
German - Basic knowledge (A1+ : 93% , A2 : 80 %)
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5. Publications

Scientific Articles

- **Napagoda**, **M.**, Rulíšek, L., Jančařík, A., Klívar, J., Šámal, M., Stará, I, G., Starý, I., Šolínová, V., Kašička, V., Svatoš, A.(2013). Azahelicene Superbases as MAILD Matrices for Acidic Analytes. ChemPlusChem 78, 937-942

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Talk Presentations

- **Napagoda, M.**, Svatoš, A. (2012). Identification of Sex Dependent Surface Lipids in *Drosophila melanogaster*. 11th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany

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- **Napagoda, M.T.**, Balasuriya, B.M.G.K., Dharmaratne, H.R.W., (2005). Inhibitory activities of some plant extracts upon germination of lettuce, 61st Annual Sessions of Sri Lanka Association for Advancement of Science, Colombo, Sri Lanka

- **Napagoda, M.T.,** Dharmaratne, H.R.W., Tennakoon, S.B., (2004). Antifungal activity and free radical scavenging property of xanthones from *Calophyllum thwaitesii*. 60th Annual Sessions of Sri Lanka Association for Advancement of Science, Colombo, Sri Lanka

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Poster Presentations

- **Napagoda, M.**, Stará, I., Svatoš, A., (2013**)** 1,14-diaza[5]helicene : A novel MALDI matrix for the analysis of acidic metabolites. 46. Jahrestagung der Deutschen Gesellschaft für Massenspektrometrie, Humboldt-Universität Berlin, Berlin, Germany

- **Napagoda, M**., Svatoš, A., (2013) Exploration of low-molecular-weight metabolites by MALDI-MS: Development of novel MALDI matrices. IMPRS Evaluation Symposium 2013/ 12th IMPRS Symposium, MPI for Chemical Ecology, Jena, Germany

- Weißflog, J., **Napagoda, M.**, Svatoš, A.,(2012). New matrices for negative mode MALDI-MS in the low mass region. SAB Meeting 2012, MPI for Chemical Ecology, Jena, Germany

- **Napagoda, M.**, Stará, I., Svatoš, A., (2012). Azahelicenes as MALDI matrices for acidic analytes. Joint Conference of German and Polish Mass Spectrometry Societies, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland

- **Napagoda, M.**, Svatoš, A., (2011). Identification of Sex Dependent Lipids in *Drosophila* Fruit flies. ICE Symposium, MPI for Chemical Ecology, Jena, Germany

- **Napagoda, M.**, Svatoš, A., (2011) Development of fast screening and imaging methods for the analysis of natural products. 10th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany

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6. Awards

- International Max Planck Research School(IMPRS) fellowship for PhD studies at Max Planck Institute for Chemical Ecology/ Friedrich-Schiller-University, Jena, Germany (2010-2013)

- Hiran Tillekeratne Award for the Outstanding Postgraduate Research in Medicine -University Grants Commission, Sri Lanka (2008)

- Kandiah Memorial Graduateship Award for the Outstanding Postgraduate Research in Chemistry – Institute of Chemistry Ceylon, Sri Lanka (2006)

- Award for the Best Scientific Paper (Oral) in Medicine - Third Academic Sessions, University of Ruhuna, Sri Lanka (2006)

- Swarna Senathirajah Memorial Award for Genetics and Plant Breeding-University of Colombo, Sri Lanka (2001)

Jena, 16th September 2014

Mayuri Tharanga Napagoda

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